DISCUSSION
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The incidence of *Acanthamoeba* keratitis has increased sharply since its first description in 1973. However, the overall incidence is still low, especially considering the ubiquity of the parasite and the large number of people exposed to it. In many countries, contact lens wear is accepted as a major risk for *Acanthamoeba* keratitis (AK). However, in India AK is mostly associated with non-contact lens wearers. This suggests that there must be other risk factors involved in the development of this corneal disease.

Clinically, the features of keratitis associated with *Acanthamoeba* infection are reminiscent of those observed with *Herpes simplex* or in some instances, with fungi (Kirkness et al.). This similarity often leads to inappropriate medical interventions to treat the infection. The criteria for suspecting *Acanthamoeba* keratitis should always be considered. Isolation of *Acanthamoeba*, that may be present as trophozoites, cysts, or intermediate forms in corneal scrapes or biopsies, must be achieved in the laboratory, however, if the clinical diagnosis is to be confirmed. Fortunately, *Acanthamoeba* keratitis is not rapidly progressive when in comparison with some bacterial or fungal ulcers, and therefore it gives the practitioner a chance to intervene successfully even if symptoms and signs have been present for one or two weeks, or even one month (Moore et al.).

The results of our studies suggest that the incidence of *Acanthamoeba* keratitis in S. India is much higher than anticipated from the published case report.
The increase in cases of *Acanthamoeba* keratitis could indicate a true increase in incidence of the disease. This could result from increased prevalence of the organism, increased number of persons at risk or due to recent awareness on the diagnosis of the disease. We have no reason to suspect men are biologically at an increased risk for *Acanthamoeba* keratitis. Perhaps they are more likely to tolerate pain, disregard hygienic practice at work place or are involved in activities that increase their risk of corneal trauma or exposure to polluted water. The age and sex distribution of patients infected with *Acanthamoeba* show that this is an opportunistic pathogen with trauma as a common predisposing factor.

In the present study careful examination of the cases revealed plant material, dust and contaminated water clustering to a common source of infection in majority of the cases. The geographic distribution of *Acanthamoeba*, although thought to be ubiquitous, is not known. Soil and water sources in these areas may be more highly contaminated with *Acanthamoeba*, allowing more frequent exposure among persons living in those areas. *Acanthamoeba* keratitis being distributed among all age groups shows a true picture of an opportunistic infection. Although *Acanthamoeba* keratitis continues to be a relatively rare disease, its incidence is much higher than expected from published reports.

*Acanthamoeba* species have been isolated from fresh water, sea water, frozen swimming water, distribution (i.e., tap) water, bottled mineral water, industrial cooling water, air conditioners, air, sewage, soil, compost, chlorinated swimming pools, medicinal pools, hot tub, dental treatment units, gastric-lavage tubing, dialysis units, and contact lens cases. *Acanthamoebae* have been isolated
from vegetables, mushrooms, cultured cells, fish, reptiles birds and mammals. In humans, species of *Acanthamoeba* have been isolated from nasal cavities, throats, and intestines as well as from infected tissues, including cerebral tissue, lung tissue, skin wounds and cornea 26-31. In all these habitats, they are associated with different kinds of microbes like bacteria and fungi. Historically, Acanthamoeba keratitis has been associated with penetrating corneal trauma and exposure to contaminated water, soil or dust containing these microbes.

It is not surprising to find mixed infections in these cases of keratitis. The association with bacteria or fungi or both may help Acanthamoeba to invade the corneal tissue more efficiently. Polymicrobial infection with bacteria has been reported in up to 10% of the cases and may be more common 71.

A peak incidence of Acanthamoeba keratitis was noticed in the summer month of March – April upto July. On the other hand, there was a decline in September to December. In the dry summer months, generations of dust particles containing the cysts are more than in September to December, which are monsoon months for this part of the country.

The delay of patients reporting to the hospital was highlighted in this study. This may reflect differences in virulence between amoebic strains. As there is a diagnostic delay, it is likely that pathogens that are more virulent might have resulted in uncontrolled infection. Delay in diagnosis or by the patient therefore remains a serious factor for outcome in *Acanthamoeba* keratitis.
Although assays were performed with axenic cultures, testing can be accomplished in association with heat killed E. coli (65°C for 20 min) by using trophozoites or cysts taken directly from NNA plates. In the present study, 1N HCl (30 min) was used to purify the cysts for all the experimental work.

*Acanthamoeba* trophozoites have been shown to produce CPE on a variety of cultured mammalian cell cultures including VERO, Hep-2, MDBK, HEL, HEK, Pig kidney, HeLa, mouse melanoma, rabbit corneal cells and human corneal epithelium, in animal models demonstrated that damaged host cells acted as a food supply for *Acanthamoeba* after subcutaneous injection (Wilson).

Swiss white mice were used to study the pathogenicity of *Acanthamoebae*. The amoebae instilled through the nostril would bring about the death or symptoms of illnesses. It was also demonstrated that some of the strains of *Acanthamoeba* were not pathogenic by mouse pathogenicity test. In the present study also some of the strains were not pathogenic to mice.

An animal model of *Acanthamoeba* keratitis would be useful tool to examine possible risk factors, pathophysiology, immunology and therapy for this disease. Badenoch *et al.* reported a model of *Acanthamoeba* keratitis in proton rats where induction was dependent on coinoculation with bacteria. Experimental *Acanthamoeba* keratitis was induced in Wistar rats by intrastromal inoculation of *Acanthamoeba*. A porcine model of *Acanthamoeba* keratitis is strikingly similar to those present in human counterparts. Stopak *et al.* have suggested that pathogenesis of *Acanthamoeba* keratitis can follow two pathways.
pathway is restricted to the epithelium without stromal involvement and has a good prognosis. The second pathway culminates in the parasites entering the stroma resulting in extensive necrosis, edema, and infiltration of polymorphonuclear leukocytes. Others have also suggested that the most severe pathological effects of *Acanthamoeba* keratitis occur in the stroma.

Affinity column removal of collagenase eliminated the pathogenicity of the soluble parasite product and thereby confirmed the role of collagenase in producing corneal lesions in vitro\(^{109}\). These results further demonstrated that the parasite-derived collagenase could either directly or indirectly stimulate the migrations of the neutrophils. Elastase activity has also been demonstrated in *Nagleria* and *Acanthamoeba* species\(^{110}\).

The value of animal model of *Acanthamoeba* keratitis is attested to by the gravity and increasing prevalence of clinical disease in Humans.

In experimental keratitis using rat model Larkin *et al.*,\(^{114}\) identified a dynamic process in which the inflammatory response altered with time. In inflammatory response, the cell population was observed to be entirely neutrophil on day 1, with macrophages becoming predominant cell type by day 7. T cells, including activated T cells, were constituents of the immune response after the first week; B cells were conspicuously absent. The clinical severity was maximal in the first week. Destruction of amoebae was observed in sections at various times but was not quantified. Chinese hamster model for *Acanthamoeba* keratitis closely resembled the acute stages of human counter part including epithelial
ulceration, corneal opacity, edema, neutrophilic infiltration, and neovascularization (Klink et al.\textsuperscript{116}).

In the present study, a reproducible model for \textit{Acanthamoeba} keratitis was established. All Rabbits that received intra-corneal injection of \textit{Acanthamoeba} cyst suspension developed keratitis on day 2 as described by Thomas \textit{et al.}\textsuperscript{148}. The ability to isolate \textit{Acanthamoeba} from corneal scrapings on NNA and the absence of bacteria strongly suggest that the keratitis formed in this Rabbit model was exclusively caused by \textit{Acanthamoeba}.

On the third day central infiltrates were clearly visible in the experimental eye. The opaque infiltrates enlarged till day 6 and, they were characterized by feathery borders as described by Mary \textit{et al.}\textsuperscript{149}. After the day 6 there was a decline in the infection index till day 14 then a characteristic plateau was reached in all the three Groups of \textit{Acanthamoeba}. The rabbit model mimics the clinical features observed in human infection such as corneal vascularization and diffused corneal opacity formation rather than formation of an abscess. Based on the size and progression of the lesions produced the strains were grouped as Highly virulent, Moderately virulent and Less virulent groups. Highly virulent groups can produce a progressive infection in man. Whereas moderately or less virulent groups may produce infection in association with other microbial pathogens. A detailed information regarding the associated bacteria/fungal pathogens with the \textit{Acanthamoeba} virulence group was not available for all the strains. Hence, we could not correlate the virulence group with associated
pathogen. More work is required in this regard with careful correlation in clinical cases.

The histopathological studies in experimental *Acanthamoeba* keratitis reveal disruption and necrosis of stromal lamellae and invasion of the amoebae in the deep stroma with infiltrates of neutrophils. In some cases, there is an added lymphocyte component\textsuperscript{70}. Evidence suggests that sensitized T cells and macrophages play a functional role by releasing lymphokines that activate the neutrophils, an essential step in the process by which leukocytes confine and kill the amoebae\textsuperscript{150}.

An antibody response to *Acanthamoeba* spp. in people with respiratory problems has also been demonstrated\textsuperscript{105}. An Immuno Fluorescence (IF) study by *Samuel et al.* demonstrated antibody titers of 1:16 to 1:512 on ocular tissue sections from an enucleated eye after *Acanthamoeba* infection. Titers of antibody to *Acanthamoeba* between 1:20 to 1:80 was demonstrated by *Cursons et al.* by IF from human serum, the antibodies were mainly of the IgA and IgG isotypes\textsuperscript{112}. Precipitin antibodies have also been demonstrated in the serum of patient suffering with *Acanthamoeba polyphaga* keratitis\textsuperscript{4}.