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Review Article

Experimental models of *Acanthamoeba* keratitis

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ABSTRACT

Acanthamoeba keratitis (AK) is a severe and potentially blinding corneal infection caused by the protozoan *Acanthamoeba*. Despite its rare occurrence, AK poses significant challenges in diagnosis, treatment, and management due to its complex pathogenesis and resistance to conventional therapies. Experimental models have played a crucial role in deepening our understanding of the disease and developing novel therapeutic strategies. This abstract review the various experimental models utilized to study *Acanthamoeba* keratitis. These models encompass both in vitro and in vivo systems, enabling researchers to simulate the pathogenic processes involved and evaluate potential therapeutic interventions. *In vitro*, models include cell cultures, corneal epithelial cell lines, and three-dimensional corneal constructs. These systems allow the investigation of *Acanthamoeba* adhesion, invasion, host immune responses, and drug efficacy. They provide insights into the molecular mechanisms underlying *Acanthamoeba* pathogenesis and aid in the screening of potential anti-*Acanthamoeba* agents. *In vivo* models, including animal models such as rabbits and mice, mimic the clinical manifestations of AK and provide a platform for assessing disease progression, evaluating host immune responses, and testing therapeutic interventions. These models have been instrumental in elucidating the factors influencing *Acanthamoeba* pathogenesis, including host susceptibility, immune responses, and corneal tissue interactions. Overall, experimental models of *Acanthamoeba* keratitis have significantly contributed to our understanding of the disease and provided a platform for developing and evaluating novel treatment strategies. The insights gained from these models hold promise for developing more effective therapies, aiming to improve patient outcomes and mitigate the devastating consequences of *Acanthamoeba* keratitis.

1. Introduction

Infectious keratitis is an inflammation of the cornea resulting from infections with bacteria, fungi, parasites, and viruses. It is a severe vision-impairing disease that severely affects the cornea, ultimately leading to perforation or scarring. It can be the result of direct invasion of the cornea by the pathogen or immunological damage to the cornea e.g. Lyme's disease. Infectious keratitis is becoming quite common in humans mainly due to the excessive use of contact lenses and improper handling, corneal injury, and not practicing efficient hand hygiene.[1] It has been estimated that globally more than 1.5 million people per year will develop blindness due to infectious corneal ulceration which is the fifth leading cause of blindness overall, responsible for up to 3.5% of all blind persons as of 2015.[2] This burden is contributed maximally by low-income countries.[3]

Keratitis involves an interplay between the colonization of the infectious agent and the host's response to it. This interaction of pathogen and host can be well understood in a living animal model or ex vivo. Cornea has a unique "immunologically privileged microenvironment" and these models have been proven to be useful to understand the

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mechanism of pathogenesis, disease biology, host immunity, and vaccination strategies, and provide an opportunity to develop diagnostic tests and an efficient keratitis treatment regime. However, successful establishment and standardization of the keratitis animal model largely depend on the animal chosen for the specific pathogen or the type of infectious agent. The most common infectious agents causing keratitis include bacteria like *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Streptococcus pneumoniae;* fungi mainly *Aspergillus*, *Fusarium* and *Candida* spp; parasites especially *Acanthamoeba* spp; and viruses, especially herpes simplex virus & adenovirus. For *Acanthamoeba* keratitis (AK) contact lens wearing (improper handling of the contact lens and lens product) is the most frequently observed cause; however, several other risk factors like corneal abrasions with contaminated vegetable debris, refractive surgery, and penetrating keratoplasty.[4,5] This review will help provide an overview of the different experimental models including in vivo models, and ex vivo models for study of *Acanthamoeba* keratitis.

Acanthamoeba are free-living amoebae that are commonly found in the environment, particularly in water sources such as lakes, and rivers. The infection usually affects people who wear contact lenses, particularly those who use them improperly or do not practice good hygiene. It can also occur in people who have had corneal trauma. The symptoms of AK include severe eye pain, redness, blurred vision, sensitivity to light, and excessive tearing.[6,7] The infection can progress rapidly and can cause permanent damage to the cornea if left untreated.

2. Pathogenesis of *Acanthamoeba* Keratitis

The pathogenesis of AK involves several genes that are expressed by both the host and the organism. Some of the key genes involved in the pathogenesis of AK are:

1. Cysteine protease (CP) genes: *Acanthamoeba* species express several cysteine proteases, which play a critical role in the invasion of the corneal epithelium. These proteases can degrade the extracellular matrix of the cornea, facilitating the penetration of the amoeba into the corneal tissue.[8]
2. Mannose-binding protein (MBP) genes: *Acanthamoeba* species express MBPs, which bind to mannose-containing glycans on the surface of host cells, including the corneal epithelium. The interaction between MBPs and host cells can facilitate adherence and invasion of the amoeba into the corneal tissue.[9,10]
3. Matrix metalloproteinase genes: MMPs are enzymes that can degrade extracellular matrix components, including collagen and laminin. *Acanthamoeba* species express MMPs, which can contribute to the destruction of corneal tissue during infection.[11]
4. Toll-like receptor (TLR) genes: TLRs are a family of pattern recognition receptors that play a critical role in the host immune response. *Acanthamoeba* species express lipopolysaccharide (LPS) and other pathogen-associated molecular patterns (PAMPs) that can activate TLRs and trigger an inflammatory response in the cornea.[12]
5. Interleukin (IL) genes: ILs are cytokines that play a key role in the regulation of the immune response. During AK, the expression of various ILs is upregulated in the cornea, contributing to the inflammatory response and tissue damage.[13]

Overall, these genes along with other confounding factors contribute to the complex interplay between the host and the *Acanthamoeba* organism, which results in the development of AK. Understanding the exact role of these genes as well as other unexplored genes in the pathogenesis of AK could lead to the development of new therapies for this serious infection.[14–17]

3. Experimental Models available for *Acanthamoeba* Keratitis

Experimental models play a crucial role in advancing our knowledge of *Acanthamoeba* keratitis by providing a controlled and reproducible platform for investigation. These models, which can be both in vivo and ex vivo, allow researchers to study various aspects including host-parasite interaction, pathogenesis, and immune response. This review will explore the different experimental models available for the AK. By examining the strengths and limitations of each model, and considering factors such as reproducibility, ethical considerations, physiological relevance, and translational potential, we can gain a comprehensive understanding of their utility in advancing our knowledge of AK.

3.1. In vivo models

In vivo, models are best used to study the salient features of keratitis and pathogenicity inflicted by both the infectious agent and the host immune response. While the most common animal model is the mouse, other animals like rats, rabbits and hamsters have also been used. While establishing keratitis in the animal model, different routes of inoculating the organism are used that allow for direct exposure of the organism to the cornea and the establishment of infection (Figure 1).

1. Contact lens-mediated infection: Contact lens-mediated inoculation is a widely used method for establishing AK in animal models.[9,10,18–41] This technique involves placing a contact lens on the cornea and introducing *Acanthamoeba* onto the surface of the contact lens, allowing more time for the organism to

adhere to the cornea and initiate infection. However, special contact lenses are to be manufactured (which are quite expensive) to fit the mouse cornea which is small and has an acutely steep curvature. To overcome this issue, we have recently suggested the use of Parafilm as a convenient, cheaper and reliable alternative to contact lenses.[21]

Some of the advantages of using contact lens-mediated inoculation for AK in animal models are:

(a) Mimics human infection: *Acanthamoeba* keratitis in a human occurs in contact lens wearers due to the presence of the organism on contaminated lenses. The use of contact lens-mediated inoculation is one of the best ways to replicate the natural route of infection and closely mimic the conditions that lead to AK in humans.
(b) Controlled infection duration: With this method, the contact lens can be worn for a specific duration, allowing researchers to control the length of exposure to *Acanthamoeba*. This is particularly useful when investigating the different stages of infection or evaluating the effectiveness of interventions at various time points.
(c) Enhanced adherence of *Acanthamoeba*: *Acanthamoeba* has a propensity to adhere to the surface of the contact lens surface, making this method particularly effective for establishing infection. The contact lens provides a surface for *Acanthamoeba* to attach to, increasing the likelihood of successful colonization and subsequent infection.
(d) Reproducibility: Contact lens-mediated inoculation offers high reproducibility, allowing consistent and standardized infection models. The inoculum size and contact lens parameters, such as material and fit, can be carefully controlled, resulting in reliable and comparable outcomes across different experiments.
(e) Non-invasive technique: In comparison to invasive methods like intrastromal injections, contact lens-mediated inoculation is less invasive and causes minimal trauma to the cornea. This is beneficial for minimizing tissue damage, maintaining ocular integrity, and reducing potential confounding factors associated with more invasive procedures.

Usually, tarsorrhaphy is performed after contact lens-mediated infection to keep the organism-laden lens onto the cornea for a long duration. Tarsorrhaphy is a surgical procedure where the eyelids are partially or completely sewn together to partially or completely close the eye. While tarsorrhaphy can be important in certain eye conditions, its role in establishing AK in animal models is secondary to the primary methods used to stimulate the infection.

2. Intrastromal injection: In this method, a small amount of the inoculum is injected directly into the corneal stroma, using a fine needle. The procedure involves first creating a tunnel through the epithelium with a 30G needle, and then delivering the inoculum directly into the underlying stroma using a 33G Hamilton syringe that has been placed into the tunnel. This method ensures that a specific number of organisms are delivered directly into the stroma and remain confined within the stroma. However, a major limitation of this method is the thin corneal thickness of the mouse which becomes the leading cause of corneal perforation in up to 15% of cases. This method is commonly used to establish bacterial, fungal, viral or parasitic keratitis.[22,24,37,38,42–62]
3. Scratch injury: This method involved creating a small scratch or abrasion on the surface of the cornea, followed by placing the inoculum on the scratched area. The method of scratch injury and topical inoculation aims to introduce the infectious organism onto the cornea to initiate the infection process. The specific details of the corneal scratch and topical inoculation methods can vary depending on the infectious organism being studied, the desired severity of keratitis, and the specific experimental protocol. This method offers several advantages for inducing keratitis in animal models:

   (a) Stimulates natural infection: By directly introducing the infectious organism onto the cornea, these methods closely mimic the natural route of infection that occurs in human keratitis. This in turn helps to study the disease progression, host-pathogen interactions, and immune responses in a manner that closely resembles the clinical scenario.
   (b) Reproducibility: This method provides a high level of reproducibility as they allow for precise and consistent delivery of the infectious organism onto the cornea. This is essential for conducting experiments with multiple animals and comparing results across different studies.
   (c) Reduced invasiveness: Compared to more invasive methods, such as intrastromal injections or subconjunctival injections, corneal scratch and topical inoculation are relatively less invasive. This helps minimize the potential for tissue damage and adverse effects associated with the procedure itself.

4. Subconjunctival injection: In this method, the organism is injected under the conjunctiva, the thin

membrane that covers the white part of the eye and lines inside the eyelids. This method ensures that the organism is introduced close to the cornea, facilitating its migration and subsequent infection. The route of subconjunctival injection aids in the establishment of infection by providing the opportunity for *Acanthamoeba* to infiltrate the corneal tissue. These methods may be modified or combined depending on the specific organism being studied and the desired outcome of the experiment.[37]

These methods typically involve less discomfort and trauma for the animals compared to more invasive techniques. This aligns with the principles of animal welfare and reduces the potential for unnecessary suffering during the experimental process.

Overall, corneal scratch and topical inoculation methods offer researchers the ability to establish keratitis in animal models in a controlled and reproducible manner, facilitating investigations into the pathogenesis, immunology, and therapeutic interventions for this ocular disease.

3.1.1. Mice

Mice have been used for the development of various keratitis models using different modes and methods of application of the pathogen. There are numerous strains of inbred and outbred mice, genetically modified mice and a wide array of mouse-specific reagents available for experimental work. The most widely used inbred mouse strains are BALB/c and C57BL/6, and several distinctions between these backgrounds should be taken into account when designing an experiment. Some mouse strains are more likely than others to experience ocular irritation. For instance, IL-12-mediated corneal damage affects Th1-responder strains like C57BL/6N, but Th2 responders like BALB/c mice exhibit IL-18-mediated less corneal infrastructure destruction. As with age, experimental keratitis models have been employed with both younger (weeks dimensions) and older (months dimensions) mice.[63–69] Beyond those factors, the technology used, the choice of the pathogen, and its emphasis on infection are crucial. Unlike bacterial and fungal keratitis, very few studies are reported that have employed mice for the establishment of amoebic keratitis. Ren and Wu produced *Acanthamoeba* keratitis in rats and mice for comparing the advantages of three different modes of inoculation, namely, the use of intrastromal injection, the use of contact lens, and topical application of parasitic suspension, post-corneal debridement. [25] Corneal scarring alone has the lowest infection rate while scratching and then covering with contaminated contact lenses has a moderate rate of infection.[20,24] Reports suggest the use of contact lenses prepared from filter paper and parafilm as an alternative to conventional contact lenses for establishing *Acanthamoeba* keratitis in mice.[21,23] Additionally, the *Acanthamoeba* keratitis in a C57BL/6 mouse model helped elucidate that IL-17A production plays a vital role in host protection against invading parasites.[22]

3.1.2. Rat

The use of a mouse in the establishment of the keratitis model has one major shortcoming of the tiny eye size. The majority of studies on the standardization of the *Acanthamoeba* keratitis model has mainly employed the use of Wistar rats in comparison to the use of mouse and has used the technique of abrasing the cornea before the intrastromal inoculation of *Acanthamoeba* trophozoites.[44,46,49,51] Though intrasomal inoculation has a higher chance of establishing keratitis, however, has the drawback that it does not mimic the natural route of *Acanthamoeba* infection in humans, which is generally through a contact lens.[70] There are several reports related to the induction of AK in rats using the intrastromal route of *Acanthamoeba* inoculation. The role of TLR-4 has been investigated in Wistar rats during *Acanthamoeba* infection and TLR-2 and TLR-4 were found upregulated during *Acanthamoeba* infection, thereby providing a better insight into the mechanism of innate immunity.[48] Furthermore, compounds and drugs such as Propolis, Polyhexamethylene biguanide, Chlorhexidine gluconate, Neosporin, Miltefosine, and Voriconazole have been tested for their *anti-Acanthamoeba* activity in the rat keratitis model.[43,45,47,50] Another study by Zorzi et al. comprised Box Behnken design of siRNA-loaded liposomes which were used to treat *Acanthamoeba* keratitis in a murine model. The only treatment regime led to a 60% reversal of keratitis-associated corneal damage, demonstrating an integral epithelium without lymphocytic infiltrate.[61]

3.1.3. Rabbit

Rabbits are extensively used in keratitis models as they are relatively a preferable choice because their cornea resembles more like that of humans in comparison to those of other animals; they have big eyes, and the disease score can be given to a maximum of 28 in contrast to mice where the lesions can be scored to a value of 4 only.[57]

Few studies are reported in the past related to the development of AK in a rabbit model. *Acanthamoeba* keratitis was established in New Zealand white rabbits using intra-stromal injection and micro-injection of *Acanthamoeba* trophozoites to portray a natural mode of infection, unlike the one set by using an infected contact lens.[57] The authors suggest this alternate mode as *Acanthamoeba* keratitis has also been reported in non-contact lens wearers due to some corneal injury or cataract surgery. Thus, they have attempted to establish keratitis by microinjection of *Acanthamoeba* trophozoites in the anterior part of the corneal stroma. Another study has established *Acanthamoeba* keratitis by employing a contact lens with a modification of cornea debridement using a

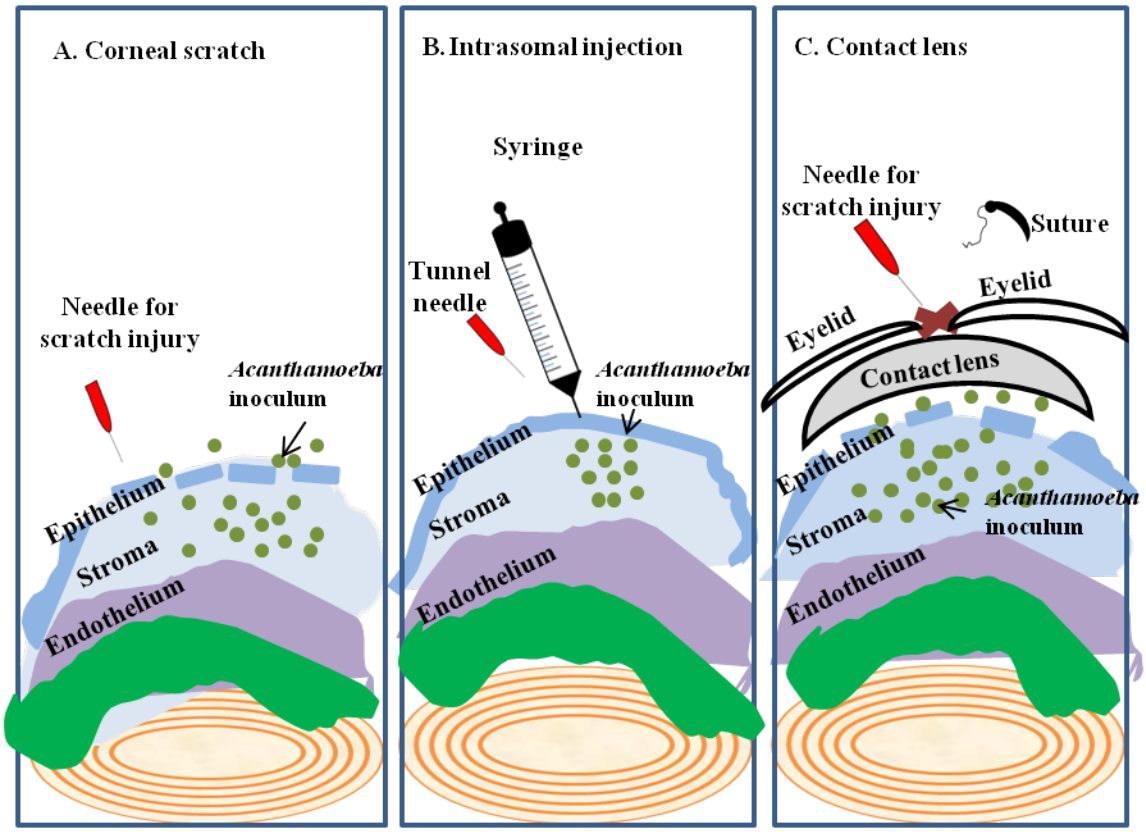

Figure 1: Schematic depiction of the different inoculation methods for inducing *Acanthamoeba* keratitis

sharp diamond burr instead of a syringe needle or surgical blade.[40] A group led by Nakagawa has demonstrated that bacteria might be contributing factors in the development of *Acanthamoeba* keratitis. Their subsequent experiments suggest that a certain number of bacteria are a critical requirement for the successful establishment of *Acanthamoeba* keratitis.[56,58] The inoculation of rabbit cornea with *Acanthamoeba* plus high P. aeruginosa treated immediately with levofloxacin, and betamethasone sodium phosphate demonstrated AK infection. This confirmed the capability of topical corticosteroids in aggravating *Acanthamoeba* keratitis infection.[55] There are reports of treating fungal keratitis with corneal cross-linking (CXL); however, (CXL; riboflavin/ultraviolet A) was found ineffective in decreasing the intensity and severity of *Acanthamoeba* keratitis.[59] In contrast, rose bengal (RB) mediated photodynamic antimicrobial therapy (PDAT) and cationic chlorin derivative photosensitizer) - mediated photodynamic antimicrobial chemotherapy (PACT) was found to be effective against the *Acanthamoeba* keratitis rabbit model.[38,39]

3.1.4. Hamster

The results from various experiments on different vertebrate animals have suggested hamsters as potent candidates for multiple procedures. The keratitis studies on hamsters have either used the contact lens laden with a pathogen or employed intra-stromal inoculations. Research in the past has suggested the cornea of hamsters is quite susceptible to *Acanthamoeba* infection based on in vitro results.[71] Studies led by Hurt et al. and others have established *Acanthamoeba* keratitis in hamsters using contact lenses laden with trophozoites on the cornea that has been abraded.[30] Another study demonstrated that *Acanthamoeba* keratitis is significantly affected by mannosylated proteins present on the ocular surface, which activate amoeba to produce pathogenic protease. This 133 kDa protease, in turn, led to the degradation of corneal epithelium and increased disease severity. Oral immunization of Chinese hamsters with recombinant mannose-binding protein was confirmed by the presence of anti-MBP in the tear fluid of immunized animals.[10] Another group led by Tripathi et al., 2013 elucidated that cytosolic phospholipase (cPLA2α) is involved in MIP-133-induced apoptosis of corneal epithelial cells and targeting cPLA2α through inhibitors can be used as a therapeutic target in *Acanthamoeba* keratitis.[26]

Additionally, *Acanthamoeba* keratitis in hamsters to study the role of macrophages and neutrophils in the disease was also attempted. Inhibition of neutrophil migration by

injecting an antibody against macrophage inflammatory protein 2 (MIP-2) resulted in increased disease severity. Neutrophils play an essential role in fighting against *Acanthamoeba* infections in the cornea.[28,31] The intra-corneal injection of latex beads demonstrated resistance to *Acanthamoeba* keratitis, most likely mediated by macrophages. Furthermore, the treatment of macrophages with the macrophagicidal drug clodronate eliminated the latex beads' protective effect.[29]

The *Acanthamoeba* infection is exacerbated under the influence of steroid treatment usually prescribed for corneal inflammation, administered after surgery, or to prevent corneal graft rejection. McClellan et al. have suggested effective amoebicidal therapy while taking topical steroidal treatment for *Acanthamoeba*.[32] The role of TLR-4 in disease pathogenesis and a potential drug target for devising better treatment options for *Acanthamoeba* infections has been explored previously.[25] Alexedine and miltefosine have shown effective results against *Acanthamoeba* keratitis.[47,72] However, the assessment of riboflavin and UV-A light treatment against *Acanthamoeba* did not demonstrate any anti-trophozoite activity.[27]

3.1.5. Other animals

The *Acanthamoeba* keratitis model has been established maximally in mice, rats, rabbits and hamsters. A group led by He et al. had successfully developed the *Acanthamoeba* keratitis model in Yucatan micropigs using the route of parasite-laden contact lenses. They suggested a strong correlation between the clinical and histopathologic features of contact lens-induced AK in pigs as well as the anatomical similarity of the pig eye to the human eye.[41]

3.2. Ex vivo AK model

To study AK ex vivo, which means outside of a living organism, researchers typically use cell culture techniques. Cell culture models involve growing cells from the cornea, which is the outermost layer of the eye, in a laboratory dish and exposing them to *Acanthamoeba*. This can be useful for understanding the basic biology of the interaction between the amoeba and the cornea cells, but it may not completely replicate the complex environment of the eye. The potential of *Acanthamoeba* castellani to adhere, permeate, and damage healthy, intact corneas of 11 mammalian and one avian species was examined in a series of in vitro investigations. It was observed that the parasite failed to produce any significant cytopathic effects on mice, rats, cotton rats, horses, guinea pigs, cows, chickens, dogs, and rabbits. However, during the 24-hour in vitro incubation phase, parasites attached, penetrated, and severely damaged the corneas of humans, pigs, and Chinese hamsters. The findings show that A. castellani exhibits strict host specificity at the surface of the host cell.[71] The ex vivo models have a set of advantages and limitations, and researchers often use a combination of approaches to better understand the biology of *Acanthamoeba* and develop new treatment options.

4. Limitations of Animal Models

Animals have been the most frequently used models to study diseases of humans including keratitis. However, there are some characteristics which are uniquely different in the human eye compared to animals. The human cornea is about 11.5 mm in diameter while that of rabbits and mice have corneas of 13 and 2.5-3.5mm respectively. Humans have thicker corneas than rabbits and mice, and their blink intervals are about 2.8 seconds compared to nearly 30 seconds for both of the other species.[70,73,74] The composition of lacrimal gland secretions is also different with an abundance of lysozyme in human secretions. These factors affect the adherence and invasion properties of pathogens and also the host defence. Despite various contrasting characteristics between humans and animals that primarily affect the results obtained from animal models, animals have conferred the basic understanding of disease pathogenesis, and pathology, and finding drugs and compounds effective against infectious keratitis.[75–80]

1. Species differences: Animals used in keratitis models may exhibit variations in ocular anatomy, physiology, and immune responses compared to humans. These differences can influence the course and characteristics of the disease, potentially limiting the direct applicability of findings to human keratitis.
2. Lack of host immune system diversity: Animal models often involve studying keratitis in a specific strain or species of animals with limited genetic diversity. This can restrict the ability to capture the full spectrum of host immune responses and disease outcomes observed in humans.
3. Artificial inoculation methods: The methods used to establish keratitis in animal models, such as corneal scratch, topical inoculation, or subconjunctival injection, involve artificial means of introducing the infectious organism. These methods may not fully replicate the natural infection route and dynamics observed in human keratitis.
4. Shorter disease course: The natural course of keratitis in animals may differ from the typically longer duration and chronic nature of keratitis in humans. The shorter disease course in animal models can affect the understanding of disease progression, chronicity, and long-term outcomes.

5. Conclusion

In conclusion, animal models have proven to be invaluable in providing insights into the pathogenesis, host immune response, and treatment strategies for Acanthamoeba

keratitis. Although animal models have provided significant contributions to our understanding of AK, it is important to acknowledge their limitations. Animal models do not fully replicate the complexity of the human eye and the host immune response. Furthermore, there can be variations in the response to infection among different animal species, which may limit the generalizations of the findings. These models have provided a platform for further research, enabling the development of improved diagnostic techniques, therapeutic interventions, and preventive measures to combat this sight-threatening disease.

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There is no conflict of interest

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