Acta Biochimica Indonesiana

https://doi.org/10.32889/actabioina.240

RESEARCH ARTICLE

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Efficacy of liposomal amphotericin B eye drops at two concentrations in experimental aspergillus keratomycosis in rabbits

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ABSTRACT

Background: Keratomycosis, a fungal corneal infection prevalent in tropical regions, frequently leads to visual impairment and blindness. Liposomal amphotericin B (L-AmB) offers improved efficacy and reduced toxicity compared to conventional amphotericin B deoxycholate and represents a promising option for topical ophthalmic use.

Objective: To evaluate and compare the therapeutic efficacy of L-AmB eye drops at 0.15% and 0.5% concentrations in experimental *Aspergillus* keratomycosis in New Zealand White rabbits.

Methods: Four rabbits received midstromal corneal inoculation with a mixed suspension of *Aspergillus flavus*, *A. fumigatus*, *A. niger*, and *A. terreus* (2 x 10^4 CFU/0.05 mL). L-AmB eye drops were applied hourly from day 8 post-inoculation until corneal cultures became negative. Antifungal susceptibility was assessed by disc diffusion. Clinical response was monitored by slit lamp examination using a modified scoring system.

Results: Negative corneal cultures were achieved in all treated eyes within 10 to 18 days (mean 15 days, L-AmB 0.15%) and 11 to 15 days (mean 14 days, L-AmB 0.5%). No toxic effects were observed. Complete corneal transparency without cicatrix was achieved in one eye treated with L-AmB 0.15%.

Conclusion: Both concentrations were effective and safe. Liposomal AmB 0.15% appears as efficacious as 0.5% for experimental *Aspergillus* keratomycosis. Clinical studies in humans are warranted.

Keywords: amphotericin B liposomal, *Aspergillus*, eye drops, keratomycosis, New Zealand rabbit

Introduction

Keratomycosis, a fungal infection of the cornea, represents a significant cause of ocular morbidity and preventable blindness, particularly in tropical and subtropical regions [1,2]. The global burden of fungal keratitis has been estimated at over one million cases annually, with the highest prevalence reported in South and Southeast Asia, sub-Saharan Africa, and Latin America [2,3]. Filamentous fungi, predominantly *Fusarium* species and *Aspergillus* species, account for the majority of cases in tropical settings, while *Candida* species are more frequently identified in temperate climates [3,4]. In Indonesia, fungal keratitis contributes substantially to the overall burden of serious fungal disease [5]. The condition frequently arises from micro-traumatic

corneal lesions, particularly in agricultural workers, and may progress to severe complications including endophthalmitis, corneal perforation, staphyloma, and permanent visual loss [1,2,6]. Because the disease is often diagnosed late and conventional antifungal therapy does not consistently yield satisfactory outcomes, a considerable proportion of patients ultimately require surgical intervention such as keratoplasty [7].

Among the antifungal agents available for the treatment of keratomycosis, amphotericin B (AmB) remains one of the most widely used due to its broad-spectrum activity against both filamentous and yeast-like fungi [8]. However, its clinical application is constrained by poor aqueous solubility, which necessitates the use of a solubilizer such as deoxycholate. The deoxycholate formulation is associated with considerable local toxicity, including epithelial damage and ocular surface irritation, limiting its tolerability as a topical preparation [9,10]. To overcome these limitations, lipid-based formulations of AmB were developed, initially for the treatment of systemic mycoses, and have demonstrated improved bioavailability, reduced toxicity, and superior therapeutic indices compared to the conventional deoxycholate preparation [9,11,12]. Among these, liposomal amphotericin B (L-AmB; AmBisome) has been the most extensively studied and is now recommended for the treatment of several invasive fungal infections [12,13].

The application of L-AmB as a topical ophthalmic preparation represents a promising but underexplored strategy for the management of fungal keratitis. Liposomal drug delivery systems offer several theoretical advantages for ocular use, including prolonged corneal contact time, enhanced tissue penetration, and reduced systemic absorption [14]. Liposomal AmB 0.15% has been shown to achieve effective corneal drug concentrations and to exert antifungal activity against *Fusarium solani* keratitis in an experimental rabbit model [15]. Moreover, good bioavailability of L-AmB in corneal tissue has been reported following topical application [16]. In a complicated clinical case of resistant *Fusarium solani* keratitis, topical L-AmB was used successfully in France [17], and the recent global guideline for the diagnosis and management of candidiasis broadly recommends L-AmB for ocular candidiasis [18]. However, the optimal concentration of L-AmB for topical ophthalmic use has not been established, and whether a lower concentration of 0.15% provides equivalent efficacy to the standard reconstituted concentration of 0.5% remains unknown [18].

We previously reported the pharmacokinetics and tissue toxicology of L-AmB eye drops at both 0.15% and 0.5% concentrations in the rabbit eye, demonstrating adequate corneal penetration and absence of toxic effects at both concentrations [19]. The present study follows from that work and aims to evaluate the therapeutic efficacy of L-AmB eye drops at these two concentrations in an experimental model of *Aspergillus* keratomycosis in New Zealand rabbits. Four clinically relevant *Aspergillus* species, namely *A. flavus*, *A. fumigatus*, *A. niger*, and *A. terreus*, were used to induce infection, reflecting the diversity of filamentous fungal pathogens encountered in clinical practice. The comparison of two concentrations of the same liposomal preparation was designed to determine whether the lower, potentially less toxic and more economical concentration of 0.15% is sufficient for therapeutic purposes.

Methods

Ethics statement and animals

Twelve adult female New Zealand White rabbits (body weight 2.5–3.5 kg) were used in this study. The study was approved by the Ethical Commission of the Faculty of Medicine Universitas Indonesia (No. 692/H2.F1/ETIK/2013). Animals were housed individually and maintained according to the updated Declaration of Helsinki, European Directives for Animal Experiments (1986/609/EEC and 2010/63/EU), and established guidelines for the care and use of laboratory rabbits [21,22,23]. Animals had ad libitum access to water and food, were allowed one week of acclimatization prior to any procedure, and were monitored daily for general health. Corneas were examined by slit lamp before the start of the experiment.

Study design

Of the twelve rabbits, seven were used for pharmacokinetic and toxicological studies, as reported previously [19]. The remaining five rabbits were used for the efficacy study reported here. Four of these five rabbits received midstromal corneal injections of a mixed fungal suspension to induce keratomycosis and were subsequently treated with L-AmB eye drops. Rabbits 1 and 2 received L-AmB 0.15%, and rabbits 3 and 4 received L-AmB 0.5%. The fifth rabbit served as a dual control: the right eye (oculus dexter, OD) was infected but left untreated as a positive control, while the left eye (oculus sinister, OS) remained uninfected as a negative control.

Infected rabbits were observed daily for eight days by slit lamp to confirm the development of keratomycosis, after which treatment was initiated. L-AmB eye drops were applied every hour from 07:00 to 19:00 daily, and treatment was continued until corneal cultures became negative. Rabbit 1 and rabbit 3 were euthanized on day 26; rabbit 2 and rabbit 4 were euthanized on day 32. Rabbit 5 was euthanized on day 12. Euthanasia was performed in accordance with AVMA guidelines [26].

Preparation of liposomal amphotericin B

L-AmB used in this study was AmBisome® (Gilead Sciences Ltd., Munich, Germany), purchased from a public German pharmacy. The lyophilized yellow powder (50 mg per vial) was reconstituted by adding 10 mL of sterile double-distilled water to obtain a suspension at a concentration of 5 mg/mL (0.5% L-AmB), according to the manufacturer's instructions (AmBisome® Information Sheet, Gilead Sciences Ltd., Carrigtohill, Ireland). For the 0.15% preparation, one third of the reconstituted volume was further diluted accordingly.

Both dispersions (0.5% and 0.15%) were either filtered through the Millipore 500 nm filter provided in the commercial AmBisome® package, or extruded five times through an Avestin LiposoFast device with a 100 nm polycarbonate filter, and transferred into small-volume eye-drop containers [19]. Particle size distribution and polydispersity of the resulting preparations were measured using a Particle Analyzer (Beckman Coulter) at the Nanotech Indonesia Laboratory of Badan Pengkajian Penerapan Teknologi (BPPT), and compared across three preparation conditions: without filtration, after Millipore filtration, and after Avestin extrusion (Table 1).

Preparation of fungal inoculum

Fungal suspensions were prepared in the Department of Parasitology, Faculty of Medicine, Universitas Indonesia, under laminar airflow conditions [19]. Four *Aspergillus* species were used: *A. fumigatus*, *A. niger*, *A. flavus*, and *A. terreus*. Spore counts were determined by hemocytometer and each suspension was serially diluted to a final concentration of 2 x 10^4 CFU/0.05 mL. Equal volumes of the four species suspensions were combined into a single mixed inoculum for corneal injection (Table 2).

Induction of experimental keratomycosis

Prior to inoculation, rabbits were anaesthetized by intramuscular administration of xylazine (5 mg/kg) [24] and ketamine (50 mg/kg) [25]. A volume of 0.05 mL of the mixed fungal suspension (2 x 10^4 CFU) was injected midstromally into the cornea of each eye under aseptic and antiseptic conditions. Development of keratomycosis was confirmed by daily slit lamp examination over eight days before treatment was commenced.

Antifungal susceptibility testing

Antifungal susceptibility of the four *Aspergillus* species against L-AmB was assessed by disc diffusion on Mueller-Hinton agar. Fungal suspensions at a concentration of 5 x 10^4 CFU/mL were inoculated onto the entire agar surface of 100 mm Petri dishes using cotton buds. After allowing 15 minutes for the suspension to dry, six-millimeter Whatman® test discs were placed on the inoculated surface: two discs for L-AmB 0.15%, two discs for L-AmB 0.5%, and one disc containing 20 ng standard

Table 1. Particle size analysis (PSA) of L-AmB preparations under three filtration conditions.

Preparation condition	Size range (nm)	Mean size, nm (SD)	Polydispersity index
Without filtration	30.5–57.7	48.5 (±14.7)	0.247
Millipore filter (500 nm)	1.5–34.4	22.9 (±17.2)	0.453
Avestin LiposoFast (100 nm)	27.1–35.5	31.9 (±9.3)	0.217

amphotericin B as control. Plates were incubated at 35°C for 24 hours, after which the diameter of the inhibition zone around each disc was measured with a ruler. Susceptibility was interpreted according to Clinical and Laboratory Standards Institute (CLSI) criteria [20]: sensitive if the inhibition zone diameter was ≥15 mm, intermediate if 10–14 mm, and resistant if <10 mm.

Clinical evaluation

Treated eyes were examined by slit lamp on days 1, 5, 10, 12, 15, 16, 18, 20, and 25 of therapy, and on the day of euthanasia. The positive control eye (rabbit 5, OD) was evaluated on days 1, 5, 10, and after euthanasia on day 12. Keratomycosis severity was assessed using a modified scoring system [27] encompassing conjunctival hyperemia, corneal clouding, corneal neovascularization, hypopyon level, and diameter of corneal infiltrate. Corneal cultures were obtained by scraping and the endpoint of treatment response was defined as the first day on which culture results became negative.

Measurement of amphotericin B concentrations in tissues

Amphotericin B concentrations in ocular tissues were measured by high-performance liquid chromatography (HPLC) using a Waters HPLC device. The device specifications and analytical methods have been described previously [19]. All measurements were performed at least in duplicate. Tissue concentration in the cornea is reported here as it is directly relevant to the efficacy findings; full pharmacokinetic data for all ocular tissues have been reported previously [19].

Statistical analysis

Differences in the duration of treatment until negative cultures were obtained between the L-AmB 0.15% and L-AmB 0.5% groups were compared descriptively given the small sample size (n=2 per group). No formal statistical testing was applied. Findings from the disc diffusion susceptibility test and clinical scoring are presented as observed values.

Results

Particle size distribution of liposomal amphotericin B preparations

Particle size analysis of L-AmB preparations under three conditions is summarized in Table 1. Without filtration, liposomes had a mean size of 48.5 ± 14.7 nm (range 30.5–57.7 nm) with a polydispersity index of 0.247. After filtration through the Millipore 500 nm filter (provided with the original AmBisome® package), mean particle size decreased to 22.9 ± 17.2 nm (range 1.5–34.4 nm) with a higher polydispersity of 0.453. Extrusion through the Avestin LiposoFast with a 100 nm polycarbonate filter yielded a mean size of 31.9 ± 9.3 nm (range 27.1–35.5 nm) and the lowest polydispersity of 0.217. The Millipore-filtered preparation was selected for all subsequent experiments because its amphotericin B content corresponded most closely to the concentration stated in the manufacturer's information leaflet.

Antifungal susceptibility of Aspergillus species against liposomal amphotericin B

The fungal inoculum was prepared from four *Aspergillus* species at a final concentration of 2 x 10^4 CFU/0.05 mL per species. Dilution steps for each species are presented in Table 2. Results of

Table 2. Serial dilution steps of the four *Aspergillus* species used for preparation of the mixed fungal inoculum. Values are expressed as spore counts (x 10^4 CFU/mL).

Species	Undiluted (x10^4)	Dilution I (x10^4)	Dilution II (x10^4)	Dilution III (x10^4)
A. flavus	3200	320	32	2
A. fumigatus	2370	237	24	2
A. niger	900	90	9	2
A. terreus	596	60	6	2

Dilution III of each species (2 x 10^4 CFU/0.05 mL) was used for the mixed inoculum.

Table 3. Inhibition zone diameters (mm) from disc diffusion susceptibility testing of four *Aspergillus* species against L-AmB 0.15%, L-AmB 0.5%, and standard amphotericin B after 24 hours of incubation at 35°C.

Species	L-AmB 0.15%, mm	L-AmB 0.5%, mm	Standard AmB, mm
A. flavus	12 (I)	15 (S)	16 (S)
A. fumigatus	15 (S)	15 (S)	17 (S)
A. niger	25 (S)	28 (S)	27 (S)
A. terreus	18 (S)	19 (S)	18 (S)

S: sensitive (≥15 mm); I: intermediate (10–14 mm); R: resistant (<10 mm).

disc diffusion susceptibility testing after 24 hours of incubation are shown in Table 3. *A. niger* and *A. terreus* were sensitive to both L-AmB concentrations and to standard AmB, with inhibition zone diameters ranging from 25–28 mm and 18–19 mm, respectively. *A. fumigatus* was sensitive to both L-AmB 0.15% and 0.5%, with inhibition zones of 15 mm for both, comparable to the standard AmB control (17 mm). *A. flavus* showed intermediate susceptibility to L-AmB 0.15% (inhibition zone 12 mm) but was sensitive to L-AmB 0.5% (15 mm) and to standard AmB (16 mm). Overall, the zone of inhibition diameters for both L-AmB preparations were comparable to the standard AmB control for three of the four species tested.

Induction and confirmation of experimental keratomycosis

Midstromal injection of the mixed fungal suspension into the corneas of anaesthetized rabbits successfully induced keratomycosis in all four treated eyes, with clinical signs apparent from the first day after injection. The positive control eye (rabbit 5, OD) and the uninfected negative control eye (rabbit 5, OS) are shown in Figure 1. The procedure of midstromal fungal inoculation is illustrated in Figure 2.

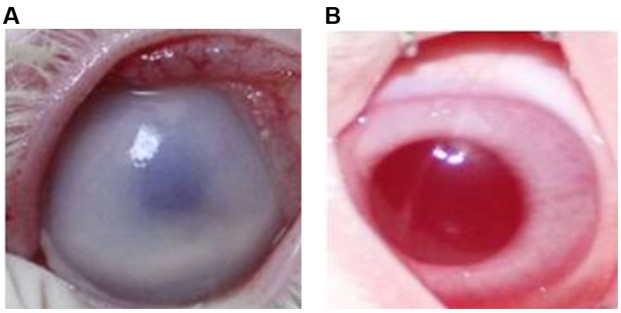

Figure 1. Slit lamp photographs of control eyes in rabbit 5 on day 1 after inoculation. (A) Positive control: right eye (oculus dexter, OD) showing corneal infiltrate following midstromal injection of the mixed *Aspergillus* suspension without subsequent treatment. (B) Negative control: left eye (oculus sinister, OS) of the same rabbit, uninfected and untreated, showing a clear and healthy cornea.

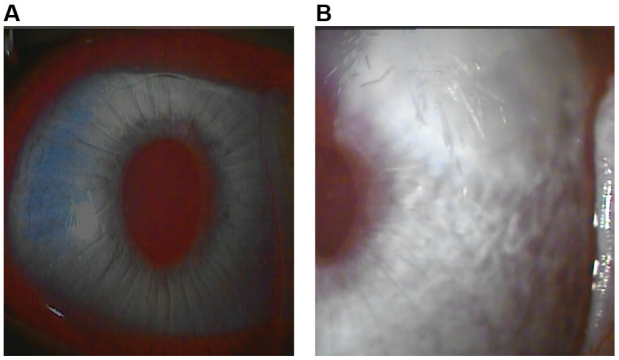

Figure 2. Midstromal injection of the fungal inoculum into the rabbit cornea. (A) Corneal appearance immediately before injection. (B) Corneal appearance immediately after midstromal injection of the mixed *Aspergillus* suspension (2 x 10^4 CFU/0.05 mL).

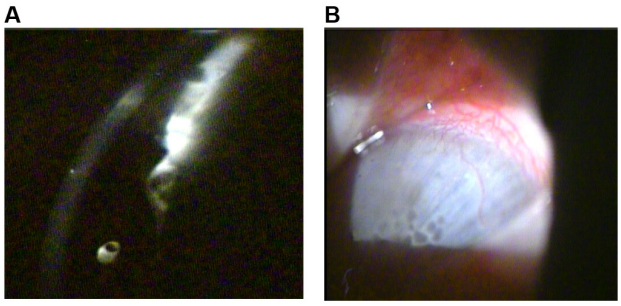

Figure 3. Slit lamp photographs of the positive control eye (rabbit 5, OD) showing disease progression without treatment. (A) Corneal infiltrate at day 10, measuring 8 x 10 mm. (B) Corneal neovascularization at day 10. By day 12, the infiltrate involved the entire corneal surface and hypopyon had reached grade 4, at which point the rabbit was euthanized.

Clinical progression in the untreated positive control

In the positive control eye (rabbit 5, OD), conjunctival hyperemia and corneal clouding increased progressively from grade 1 on day 1 to grade 3 by day 12. Corneal neovascularization remained at grade 1 throughout the observation period. Hypopyon reached grade 4 by day 12. The diameter of the corneal infiltrate was 4 x 6 mm on day 1, expanded to 8 x 10 mm on days 5 and 10, and involved the entire corneal surface by day 12, at which point the rabbit was euthanized. Corneal infiltrate progression and neovascularization in the positive control are shown in Figure 3.

Treatment outcomes: culture results and duration of therapy

Corneal cultures from all four treated rabbits (rabbits 1–4) became negative during the course of therapy. In contrast, the positive control eye (rabbit 5, OD) yielded a positive culture throughout, while the negative control eye (rabbit 5, OS) remained culture-negative. Culture negativity was achieved after 10 to 18 days (mean 15 days) with L-AmB 0.15%, and after 11 to 15 days (mean 14 days) with L-AmB 0.5% (Table 4). Individual data per eye are presented in Table 4. Given the small sample size (n=2 per group), no formal statistical comparison was performed; the difference in time to culture negativity between the two concentrations was not clinically meaningful.

Table 4. Individual and mean duration of therapy (days) until negative corneal cultures were achieved in rabbits treated with L-AmB 0.15% (rabbits 1 and 2) and L-AmB 0.5% (rabbits 3 and 4).

Group	Rabbit	Eye	Days to negative culture
L-AmB 0.15%	Rabbit 1	OD	18
		OS	10
	Rabbit 2	OD	16
		OS	16
	Mean		**15**
L-AmB 0.5%	Rabbit 3	OD	11
		OS	15
	Rabbit 4	OD	15
		OS	15
	Mean		**14**

OD: right eye (oculus dexter); OS: left eye (oculus sinister).

Clinical response to treatment: slit lamp findings and keratomycosis scoring

Treated eyes were evaluated using a modified keratomycosis scoring system [27]. Representative slit lamp photographs of the treated cornea before therapy, after one week, and after 12 days of therapy are shown in Figure 4.

Conjunctival hyperemia reached its highest level of grade 2 on day 5 in all treated eyes and decreased to zero between days 10 and 16. Corneal clouding remained at grade 1 throughout the observation period and was either grade 1 or 0 at the time of euthanasia. Hypopyon was absent (grade 0) throughout the entire treatment period in all treated eyes. Corneal neovascularization was grade 1 in the first days of observation, and had resolved to grade 0 in all treated eyes by the end of the observation period, except in one eye where grade 1 persisted until day 12. The diameter of the corneal infiltrate ranged from 4 x 6 mm to 8 x 9 mm at the start of therapy and decreased successively to 1 x 1 mm or zero by the time of euthanasia on day 26 or 32. No differences in clinical scores between the L-AmB 0.15% and L-AmB 0.5% groups were observed throughout the treatment and observation period.

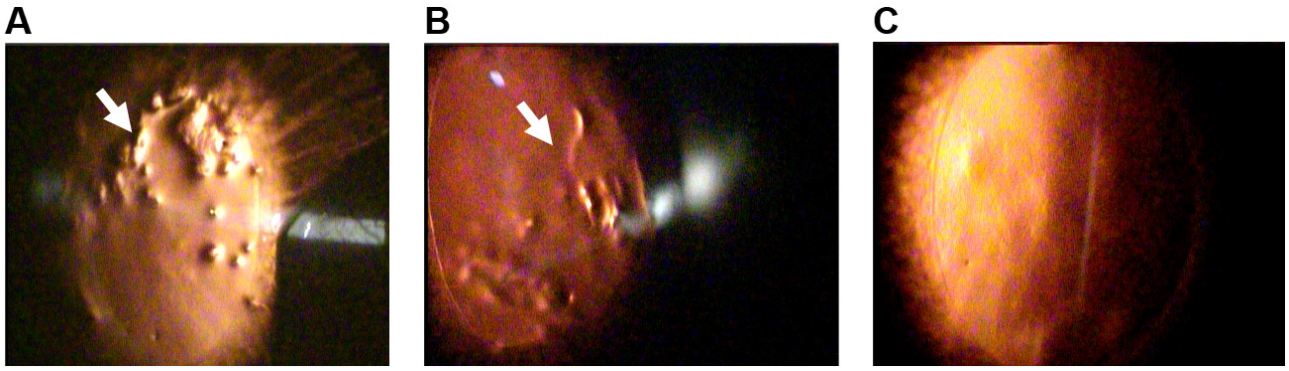

Figure 4. Slit lamp photographs of a treated eye (rabbit 2, left eye, OS, L-AmB 0.15%) showing clinical response to therapy. (A) Before therapy (day 0 of treatment, day 8 after inoculation): corneal infiltrate measuring 8 x 9 mm with conjunctival hyperemia grade 2. (B) After one week of therapy (day 7): reduction in infiltrate size and hyperemia. (C) After 12 days of therapy (day 12): further reduction in corneal infiltrate with clearing of hyperemia.

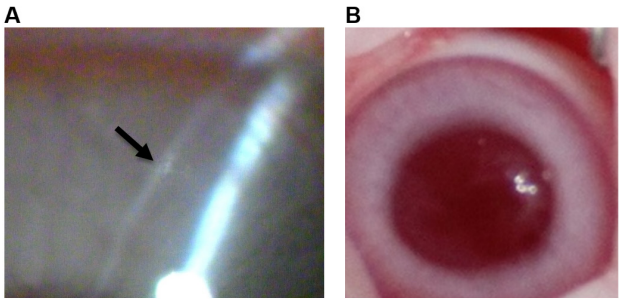

Figure 5. Slit lamp photographs of both eyes of rabbit 1 at the time of euthanasia (day 26), following treatment with L-AmB 0.15%. (A) Right eye (OD): corneal cicatrix visible as a faint opacity at the site of the former infiltrate. (B) Left eye (OS): cornea entirely clear with no cicatrix formation, representing the only eye in this study that achieved complete corneal transparency after treatment.

Corneal cicatrix formation after treatment

At least slight corneal cicatrix formation was observed in all treated eyes after completion of therapy. An exception was noted in the left eye (OS) of rabbit 1, treated with L-AmB 0.15%, in which the cornea remained clear without any visible cicatrix at the time of euthanasia. The right eye (OD) of the same rabbit showed visible cicatrix formation, illustrating the between-eye variability even within the same animal and at the same concentration. Representative photographs are shown in Figure 5.

Amphotericin B tissue concentrations in the cornea

Complete pharmacokinetic data for all ocular tissues following three days of L-AmB eye drop administration have been reported previously [19]. In the present study, corneal tissue was additionally sampled at the time of euthanasia from rabbits treated for the efficacy study, providing concentration data after prolonged treatment. Amphotericin B concentration in the cornea was 0.08 ± 0.06 µg/mL on day 26 and 0.12 ± 0.06 µg/mL on day 32 of treatment. These values indicate that measurable AmB concentrations were maintained in corneal tissue throughout the extended treatment period, consistent with the sustained antifungal activity observed clinically.

Discussion

The main finding of this study is that liposomal amphotericin B eye drops at both 0.15% and 0.5% concentrations were effective in eradicating experimental *Aspergillus* keratomycosis in New Zealand rabbits, with negative corneal cultures achieved in all treated eyes within 10 to 18 days of therapy. No clinically meaningful difference in time to culture negativity was observed between the two concentrations (mean 15 days for 0.15% vs. 14 days for 0.5%), and clinical scoring parameters followed a comparable course in both groups. Notably, the only eye that achieved complete corneal transparency without cicatrix formation after treatment was treated with L-AmB 0.15%, further supporting the adequacy of this lower concentration for therapeutic purposes.

Regarding the liposomal preparation, the Millipore-filtered dispersion was selected for the

experiments because its amphotericin B content corresponded most closely to the manufacturer's specifications. However, it is noteworthy that this preparation yielded the highest polydispersity index (0.453) despite producing the smallest mean particle size (22.9 ± 17.2 nm). A high polydispersity index indicates a heterogeneous distribution of particle sizes within the preparation, which may have implications for dose consistency and corneal penetration, as particles of varying sizes may differ in their ability to traverse corneal epithelial barriers and interact with target tissues [14]. In contrast, the Avestin LiposoFast extrusion yielded a more homogeneous preparation with a lower polydispersity index of 0.217 and a narrower size range. For future ophthalmic applications, preparations with lower polydispersity may offer more predictable pharmacokinetic behavior, and this warrants consideration in the design of purpose-built liposomal formulations specifically designed for the eye [14,30].

The pharmacokinetic data from this study showed that measurable amphotericin B concentrations were maintained in corneal tissue throughout the extended treatment period, at 0.08 ± 0.06 µg/mL on day 26 and 0.12 ± 0.06 µg/mL on day 32. These values are consistent with the good corneal bioavailability of topically applied L-AmB previously reported by Ghosh et al. [16] and are complementary to the tissue concentration data from our earlier three-day pharmacokinetic study [19]. The sustained presence of AmB in corneal tissue during prolonged treatment supports the rationale for continued topical application until culture negativity is confirmed, and is consistent with the progressive clinical improvement observed in all treated eyes.

No toxic effects were observed in any of the treated eyes throughout the treatment and observation period, consistent with the findings of our previous toxicological study [19] and with data reported by Kaji et al., who demonstrated that topical L-AmB 0.15% produced no toxic effects on the cornea or conjunctiva [28]. The absence of toxicity at both concentrations supports the safety of prolonged topical use of L-AmB in this model. This favorable toxicity profile stands in contrast to conventional amphotericin B deoxycholate, where the deoxycholate moiety itself is associated with epithelial damage and ocular surface irritation [9,10], and represents one of the principal advantages of the liposomal formulation for ophthalmic application.

The in vitro susceptibility results require careful interpretation in relation to the in vivo outcomes. *A. flavus* showed only intermediate susceptibility to L-AmB 0.15% (inhibition zone 12 mm) compared to sensitivity for L-AmB 0.5% (15 mm) and standard AmB (16 mm). Despite this finding, L-AmB 0.15% achieved culture negativity in vivo within a clinically comparable timeframe to L-AmB 0.5%. This apparent discrepancy between in vitro and in vivo results may be explained by several factors. First, the liposomal formulation enhances corneal drug retention and contact time compared to conventional preparations, providing sustained local drug concentrations that may overcome borderline in vitro susceptibility [15,16]. Second, the use of a mixed inoculum of four *Aspergillus* species means that the in vivo outcome reflects the collective response of all species present, and the three fully sensitive species may have predominated in determining the overall culture result. Third, disc diffusion is a semi-quantitative method that may not fully capture the dynamic interaction between liposomal drug delivery and fungal cells in tissue. These considerations suggest that intermediate in vitro susceptibility of *A. flavus* to L-AmB 0.15% does not necessarily predict in vivo treatment failure, though this relationship warrants further investigation with minimum inhibitory concentration determination and species-specific infection models.

The use of a mixed inoculum comprising four *Aspergillus* species was intended to reflect the diversity of filamentous fungal pathogens encountered in clinical practice in tropical settings. However, it also introduces a limitation in that the relative contribution of each species to the infection and to the treatment response cannot be determined from culture results alone. Future studies using species-specific infection models would allow more precise characterization of the

efficacy of L-AmB against individual *Aspergillus* species, particularly *A. flavus* given its intermediate in vitro susceptibility profile observed here.

Liposomal AmB 0.15% (AmBisome®) has previously been shown to inhibit the clinical progression of *Fusarium solani* keratitis and to sterilize corneal cultures within five days in an experimental rabbit model using the same commercial preparation [15]. However, that study employed a short treatment course of only five days and did not assess long-term corneal resolution; the reported outcome therefore reflects clinical improvement and microbiological clearance rather than complete healing. The present study extends these observations by demonstrating sustained fungal eradication across a more prolonged treatment course, with culture negativity maintained through to euthanasia on day 26 or 32 and 100% eradication confirmed by end of treatment. Direct comparison of time to cure between the two studies is not possible given differences in pathogen species, treatment duration, and infection severity at baseline.

In an experimental *A. flavus* endophthalmitis model, the clinical outcome of intravitreal voriconazole (100 μg) surpassed that of intravitreal amphotericin B deoxycholate (5 μg), although amphotericin B reduced colony counts more effectively [29]. Inflammatory responses were reported to be milder in the voriconazole group, though it was not clarified whether the inflammation in the amphotericin B group was attributable to the deoxycholate vehicle rather than to amphotericin B itself [29]. The model of topical keratomycosis used in the present study differs fundamentally from intravitreal administration for endophthalmitis, and the use of a liposomal rather than deoxycholate formulation eliminates the vehicle-related toxicity concern, making direct comparison difficult. Nevertheless, these findings collectively underscore the importance of both drug selection and formulation vehicle in the management of ocular fungal infections.

Liposomal AmB has been used successfully as topical eye drops in a clinically resistant case of *Fusarium solani* keratitis [17], and a clinical study at a tertiary eye care center demonstrated comparable efficacy of L-AmB across three different formulations in patients with fungal keratitis, including cases caused by *Aspergillus flavus* [18]. The recent global guideline for the diagnosis and management of candidiasis also broadly recommends L-AmB for ocular candidiasis [13]. The present study adds experimental evidence supporting the efficacy of topical L-AmB against *Aspergillus* keratitis. Purpose-built nanocarrier-based amphotericin B delivery systems designed specifically for ophthalmic use are under active development [30] and may offer further improvements in drug stability, corneal penetration, and polydispersity control compared to the repurposed parenteral formulation evaluated here.

The main limitations of this study are the small number of experimental animals (n=2 per treatment group), which precludes formal statistical analysis and limits the generalizability of the findings, and the use of a parenteral liposomal preparation not originally designed for topical ophthalmic use. The Millipore-filtered preparation, while consistent with manufacturer specifications, exhibited a relatively high polydispersity index that may affect the reproducibility of drug delivery to the cornea. The cost of lipid-based amphotericin B formulations also remains a barrier to widespread use in resource-limited tropical settings where the burden of fungal keratitis is highest [5,10]. Alternative approaches, including less expensive antiseptic compounds [31] and micro-invasive pharmacotherapy such as intracorneal microneedle delivery of amphotericin B [34], merit consideration alongside advanced liposomal formulations, while surgical options such as therapeutic keratoplasty [32,33] or conjunctival flap procedures combined with lamellar keratoplasty [35] remain reserved for the most severe cases refractory to medical therapy.

Conclusion

Liposomal amphotericin B eye drops at both 0.15% and 0.5% concentrations were effective and safe in the treatment of experimental *Aspergillus* keratomycosis in New Zealand rabbits, achieving complete fungal eradication in all treated eyes within a maximum treatment duration of 32 days.

No clinically meaningful difference in efficacy was observed between the two concentrations, and the lower concentration of 0.15% appears to be as efficacious as 0.5% for this indication. The intermediate in vitro susceptibility of *A. flavus* to L-AmB 0.15% did not translate into reduced in vivo efficacy, likely reflecting the pharmacokinetic advantages of liposomal drug delivery in corneal tissue. These findings provide experimental support for the use of topical L-AmB in the treatment of fungal keratitis caused by *Aspergillus* species and should serve as the basis for clinical studies in humans. Future investigations should employ purpose-built nanocarrier-based amphotericin B formulations designed for ophthalmic application, which may offer superior physicochemical properties including lower polydispersity and more predictable corneal drug delivery, further improving upon the off-label use of the parenteral liposomal preparation evaluated in the present study.

Acknowledgements

The authors thank the Faculty of Medicine, Universitas Indonesia, for providing laboratory and animal facility access to conduct this study. The authors also thank the Nanotech Indonesia Laboratory, Badan Pengkajian Penerapan Teknologi (BPPT), for particle size analysis, and the Department of Parasitology, Faculty of Medicine, Universitas Indonesia, for support in fungal suspension preparation.

Funding

This study was funded by Universitas Trisakti, Jakarta, Indonesia. The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Declaration of interest

The authors declare no conflict of interest. In particular, no financial or other support was received from Gilead Sciences Ltd., Ireland or Germany.

Authors' contributions

HA: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Project administration; Writing – original draft. AE: Data curation; Formal analysis; Investigation (HPLC measurements and analytical data); Methodology; Writing – review and editing. RR: Investigation (animal housing, care, and monitoring); Resources; Writing – review and editing. RS: Funding acquisition; Resources (facilities, Faculty of Medicine Universitas Indonesia); Supervision; Writing – review and editing. RA: Investigation (fungal suspension preparation and disc diffusion susceptibility testing); Methodology; Writing – review and editing. HJF: Conceptualization; Methodology; Supervision; Validation; Writing – original draft; Writing – review and editing.

Declaration of interest

The authors declare no conflict of interest; in particular, there was no support by Gilead Sciences Ltd., Ireland/Germany, neither financially nor otherwise.

Received: February 15, 2026
Revised: March 9, 2026
Accepted: March 10, 2026
Published: March 11, 2026

References

1. Khorashad AS, Kashani M, Parandin F, et al. Prevalence of Fungal Keratitis in Suspected Keratomycosis Cases Over Five Years in Southeastern Iran. Arch Clin Infect Dis. 2025;20(6):e161256. https://doi.org/10.5812/archcid-161256
2. Brown L, Leck AK, Gichangi M, Burton MJ, Denning DW. The global incidence and diagnosis of fungal keratitis. Lancet Infect Dis. 2021;21(3):e49-e57. https://doi.org/10.1016/S1473-3099(20)30448-5
3. Hoffman JJ, Burton MJ, Leck A. Mycotic Keratitis: A Global Threat from the Filamentous Fungi. J Fungi (Basel). 2021;7(4):273. https://doi.org/10.3390/jof7040273
4. Ahmadikia K, Aghaei Gharehbolagh S, Fallah B, et al. Distribution, Prevalence, and Causative Agents of Fungal Keratitis: A Systematic Review and Meta-Analysis (1990 to 2020). Front Cell Infect Microbiol. 2021;11:698780. https://doi.org/10.3389/fcimb.2021.698780

5. Wahyuningsih R, Adawiyah R, Sjam R, et al. Serious fungal disease incidence and prevalence in Indonesia. Mycoses. 2021;64(10):1203-1212. https://doi.org/10.1111/myc.13304
6. Mahmoud YAG. Fungal keratitis efficient treatments using surface active agents (cetrimide): an overview. J Bacteriol Mycol Open Access. 2016;2(3):69-73. https://doi.org/10.15406/jbmoa.2016.02.00026
7. Sharma N, Bagga B, Singhal D, Nagpal R, Kate A, Saluja G, Maharana PK. Fungal keratitis: A review of clinical presentations, treatment strategies and outcomes. Ocul Surf. 2022;24:22-30. https://doi.org/10.1016/j.jtos.2021.12.001
8. Qasim M, Baipaywad P, Udomluck N, et al. Enhanced Therapeutic Efficacy of Lipophilic Amphotericin B Against Candida albicans with Amphiphilic Poly(N-isopropylacrylamide) Nanogels. Macromolecular Research. 2014;22(10):1125-1131. https://doi.org/10.1007/s13233-014-2162-2
9. Rodrigues CF, Henriques M. Liposomal and Deoxycholate Amphotericin B Formulations: Effectiveness against Biofilm Infections of Candida spp. Pathogens. 2017;6(4):62. https://doi.org/10.3390/pathogens6040062
10. Hamill RJ. Amphotericin B formulations: a comparative review of efficacy and toxicity. Drugs. 2013;73(9):919-934. https://doi.org/10.1007/s40265-013-0069-4
11. Lim C-b, Abuzar SM, Karn PR, et al. Preparation, Characterization, and In Vivo Pharmacokinetic Study of the Supercritical Fluid-Processed Liposomal Amphotericin B. Pharmaceutics. 2019;11(11):589. https://doi.org/10.3390/pharmaceutics11110589
12. Stone NRH, Bicanic T, Salim R, Hope W. Liposomal Amphotericin B (AmBisome®): A review of the pharmacokinetics, pharmacodynamics, clinical experience and future directions. Drugs. 2016;76(4):485-500. https://doi.org/10.1007/s40265-016-0538-7
13. Cornely OA, Sprute R, Bassetti M, et al. Global guideline for the diagnosis and management of candidiasis: an initiative of the ECMM in cooperation with ISHAM and ASM. Lancet Infect Dis. 2025;Feb 13:S1473-3099(24)00749-7. https://doi.org/10.1016/S1473-3099(24)00749-7
14. Agarwal R, Iezhitsa I, Agarwal P, et al. Liposomes in topical ophthalmic drug delivery: an update. Drug Deliv. 2014:1-17. https://doi.org/10.3109/10717544.2014.943336
15. Yavas GF, Ozturk F, Kusbeci T, et al. Antifungal efficacy of voriconazole, itraconazole and amphotericin B in experimental Fusarium solani keratitis. Graefes Arch Clin Exp Ophthalmol. 2008;246:275-279. https://doi.org/10.1007/s00417-007-0687-1
16. Ghosh AK, Rudramurthy SM, Gupta A, et al. Evaluation of Liposomal and Conventional Amphotericin B in Experimental Fungal Keratitis Rabbit Model. Transl Vis Sci Technol. 2019;8(3):35. https://doi.org/10.1167/tvst.8.3.35
17. Touvron G, Denis D, Doat M, et al. Efficacité de l'amphotéricine B liposomale dans le traitement d'une kératite à Fusarium solani résistante. J Fr Ophtalmol. 2009;32:721-726. https://doi.org/10.1016/j.jfo.2009.10.011
18. Tandon R, Lomi N, Vanathi M, Gupta N, et al. Evaluation of liposomal amphotericin B for the treatment of fungal keratitis in a tertiary eye care hospital. Indian J Ophthalmol. 2023;71(2):518-523. https://doi.org/10.4103/ijo.IJO_1474_22
19. Amalia H, Estuningtyas A, Ridwan R, Sitompul R, Adawiyah R, Freisleben HJ. Pharmacokinetic and Toxicological Studies of Liposomal Amphotericin B Eye Drops on the Rabbit Eye. Int J Pharm Res. 2020;12(4):1828-1834. https://doi.org/10.31838/ijpr/2020.12.04.261
20. Clinical and Laboratory Standards Institute (CLSI). Performance Standards for Antimicrobial Disk Diffusion Susceptibility Tests. 19th ed. Wayne, PA: CLSI; 2009. Document M100-S19.
21. Fuentes GC, Newgren J. Physiology and Clinical Pathology of Laboratory New Zealand White Rabbits Housed Individually and in Groups. J Am Assoc Lab Anim Sci. 2008;47(2):35-38.
22. Mähler M, Berard M, Feinstein R, et al. FELASA recommendations for the health monitoring of mouse, rat, hamster, guinea pig and rabbit colonies in breeding and experimental units. Lab Anim. 2014;48(3):178-192. https://doi.org/10.1177/0023677213516312
23. Warnke GR, Kluge R. Feeding concepts and methods in the laboratory animal facility and in animal experiments: Rabbits (Oryctolagus cuniculus L.) at the example of the breed New Zealand White. GV-SOLAS, Committee for Laboratory Animal Nutrition. 2015, translated 2021:1-15.
24. Akter MA, Yesmin N, Talukder MBA, et al. Evaluation of anaesthesia with xylazine-ketamine and xylazine-fentanyl-ketamine in rabbit: A comparative study. J Adv VetBio Sc Tech. 2023;8(1):38-46. https://doi.org/10.31797/vetbio.1129402
25. Henke JA, Astner S, Brill T, et al. Comparative study of three intramuscular anaesthetic combinations (medetomidine/ketamine, medetomidine/fentanyl/midazolam and xylazine/ketamine) in rabbits. Vet Anaesth Analg. 2005;32(5):261-271. https://doi.org/10.1111/j.1467-2995.2005.00242.x
26. Leary S, Underwood W, Anthony R, et al. AVMA Guidelines for the Euthanasia of Animals. 2013 ed. Schaumburg, IL: American Veterinary Medical Association; 2013.
27. Eaton JS, Miller PE, Bentley E, Thomasy SM, Murphy CJ. Slit Lamp-Based Ocular Scoring Systems in Toxicology and Drug Development: A Literature Survey. J Ocul Pharmacol Ther. 2017;33(10):707-717. https://doi.org/10.1089/jop.2017.0021
28. Kaji Y, Yamamoto E, Hiraoka T, Oshika T. Toxicities and Pharmacokinetics of subconjunctival injection of liposomal amphotericin B. Graefes Arch Clin Exp Ophthalmol.

2009;247:549-553. https://doi.org/10.1007/s00417-008-1003-4

29. Arianti A, Setiabudy R, Rozaliyani A, Siregar NC, Susiyant M. Comparison of intravitreal antifungal 100 µg voriconazole and 5 µg amphotericin B in experimental Aspergillus flavus endophthalmitis model in rabbits. Med J Indones. 2019;28:215-222. https://doi.org/10.13181/mji.v28i3.1794
30. Kumar G, Kalita AH, Ahmed J, et al. Advancing fungal keratitis treatment: transitioning from conventional amphotericin B therapy to nanocarrier-based delivery systems. Naunyn-Schmiedeb Arch Pharmacol. 2025; May 17. https://doi.org/10.1007/s00210-025-04268-8
31. FlorCruz NV, Evans JR. Medical interventions for fungal keratitis. Cochrane Database of Systematic Reviews. 2015;Issue 4:CD004241. https://doi.org/10.1002/14651858.CD004241.pub4
32. Christy JS, Mathews P, Ravisankar R, Akpek EK. Long-term outcomes of therapeutic corneal transplants performed in a tertiary eye care hospital in South India. Indian J Ophthalmol. 2023;71:3171-3177. https://doi.org/10.4103/IJO.IJO_1344_23
33. Mundra J, Dhakal R, Mohamed A, et al. Outcomes of therapeutic penetrating keratoplasty in 198 eyes with fungal keratitis. Indian J Ophthalmol. 2019;67:1599-1605. https://doi.org/10.4103/ijo.IJO_1952_18
34. Albadr AA, Tekko IA, Vora LK, et al. Rapidly dissolving microneedle patch of amphotericin B for intracorneal fungal infections. Drug Deliv Transl Res. 2022;12:931-943. https://doi.org/10.1007/s13346-021-01032-2
35. Wang YC, Wang JS, Wang B, Peng X, Xie HT, Zhang MC. Two-step strategy: conjunctival flap covering surgery combined with secondary deep anterior lamellar keratoplasty for the treatment of high-risk fungal keratitis. Int J Ophthalmol. 2023;16(7):1065-1070. https://doi.org/10.18240/ijo.2023.07.10