Original Article

Use of white light *in vivo* confocal microscopy for the detection of spatial changes in the corneal nerves in cases of early-stage *Acanthamoeba* keratitis with radial keratoneuritis

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Purpose: Radial keratoneuritis (RK) is a common feature of *Acanthamoeba* keratitis (AK). *In vivo* confocal microscopy (IVCM) is noninvasive and provides real-time images for the diagnosis of corneal diseases by allowing the visualization of corneal structures and morphologies of living organisms at the cellular level. Images of AK with RK obtained using commercial white light IVCM devices have not been frequently evaluated. In the present study, a white light IVCM device was used to evaluate the corneal findings and describe spatial changes in the corneal nerves at different depths in cases of early-stage AK with RK. **Methods:** In this retrospective, observational study, white light IVCM images focused on RK were evaluated for *Acanthamoeba* cysts/trophozoites, corneal deposits, and altered corneal nerves, with special emphasis on three-dimensional spatial changes in the corneal nerves at different depths. **Results:** Seventeen eyes of 17 patients exhibiting early-stage AK with RK were included in the study. *Acanthamoeba* cysts/trophozoites were observed in the corneal epithelium of 13 eyes and stroma of 7 eyes. Alterations in the corneal nerve morphology and density were observed from the basal epithelial layer to the stromal layer in 12 eyes. *Acanthamoeba* trophozoites were attached to the corneal stromal nerves in five eyes. **Conclusion:** These findings suggest that white light IVCM can identify consistent corneal findings, particularly spatial changes in the corneal nerves, in cases of early-stage AK with RK.

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Acanthamoeba keratitis (AK) is a vision-threatening infectious disease caused by *Acanthamoeba* protozoa.[1] After reviewing our past medical records, we incidentally discovered that spatial changes in the corneal nerves in some cases of early-stage AK with Radial keratoneuritis (RK). In the present study, we reviewed our past medical records. White light *in vivo* confocal microscopy (IVCM) was used to assess cases of early-stage AK with RK. Images were obtained at different corneal layers and assessed for the characteristic findings of brightly reflective abnormal cells, corneal deposits, and altered corneal nerve structures, with special focus on spatial changes in the corneal nerves at different depths. The anatomical relationship between the *Acanthamoeba* pathogens and the abnormal corneal nerves was also evaluated.

Methods

This retrospective, cross-sectional study included consecutive patients diagnosed with early-stage AK between May 2008 and April 2016. Informed consent was obtained from all subjects after the nature, purpose, and possible consequences of the study were explained. The appropriate ethics committee approved the research protocol and the methods used in the study adhered to the tenets of the Declaration of Helsinki for the use of human subjects in biomedical research. Seventeen eyes of 17 patients were included in this study. All patients wore contact lenses, with 13 wearing soft contact lenses and four wearing orthokeratology lenses. Corneal epithelium specimens were sent for smear and culture. The data on ophthalmological history, best-corrected visual acuity (BCVA), intraocular pressure, and slit-lamp biomicroscopic findings were collected from chart reviews.

In vivo confocal microscopy

IVCM was performed for all patients by a single examiner at the first visit. One drop of 0.5% proparacaine solution and one drop of artificial tears were instilled just before the examination. A white light IVCM device (Confoscan 3.4.1; Nidek Technologies) equipped with a standard 40× water-immersion front lens (Zeiss, Oberkochen, Germany) captured the full

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thickness of the central cornea using an automatic capture technique. Special attention was paid to RK visualization. Each examination required approximately 1 to 3 min and recorded 350 images. During each visit, the measurements were repeated three times with a 4-μm *z*-interval, followed by three times with a 1.5-μm *z*-interval.

Results

Demographics

Four men and 13 women (mean age, 20.8 ± 6.4 years) were diagnosed with early-stage AK with RK [Fig. 1]. All patients wore contact lenses, with 13 wearing soft contact lenses and four wearing orthokeratology lenses. The mean duration from onset to the initial visit was 27.2 ± 3.4 days. All cultures and smears had shown positive findings for AK. All patients were treated with oral and topical anti-amoebic agents and cured without antibiotics, anti-fungal medications, or surgical management.

In vivo confocal microscopy

Findings in the corneal epithelial layer

In the basal epithelial layer, *Acanthamoeba* cysts were observed in 10 eyes as round, hyper-reflective particles with a diameter of 10 to 20 μm [Fig. 2a], while *Acanthamoeba* trophozoites were observed in 10 eyes as amorphous, hyper-reflective, irregular, wedge-shaped structures with a diameter of 10 to 20 μm. *Acanthamoeba* pathogens in the basal epithelial layer were observed without [Fig. 2a] or with [Fig. 2b and c] attachment to the basal epithelial nerves. Morphological changes in the corneal nerves in this layer or Bowman's layer were observed in eight eyes. These corneal nerves appeared thin, fragmented, and tortuous [Fig. 2c and d], with no changes in their diameters.

Findings in the corneal stromal layer

In the corneal stromal layer, *Acanthamoeba* cysts were observed in five eyes as round, hyper-reflective particles with a diameter of 10 to 20 μm [Fig. 2e and f]. Hyper-reflective spindle-shaped structures in the stroma were found in four eyes [Fig. 2f], while hyper-reflective, activated keratocytes forming a honeycomb pattern were found in 10 eyes [Fig. 2g].

Changes in the corneal nerves in the stroma

Changes in the corneal nerve structure exhibited two different patterns. In two eyes, thin, tortuous, fragmented corneal nerves with no significant changes in their diameters were observed from the corneal basal epithelial layer to the corneal stroma [Fig. 2c, d and h]. In five eyes, on the other hand, the thin,

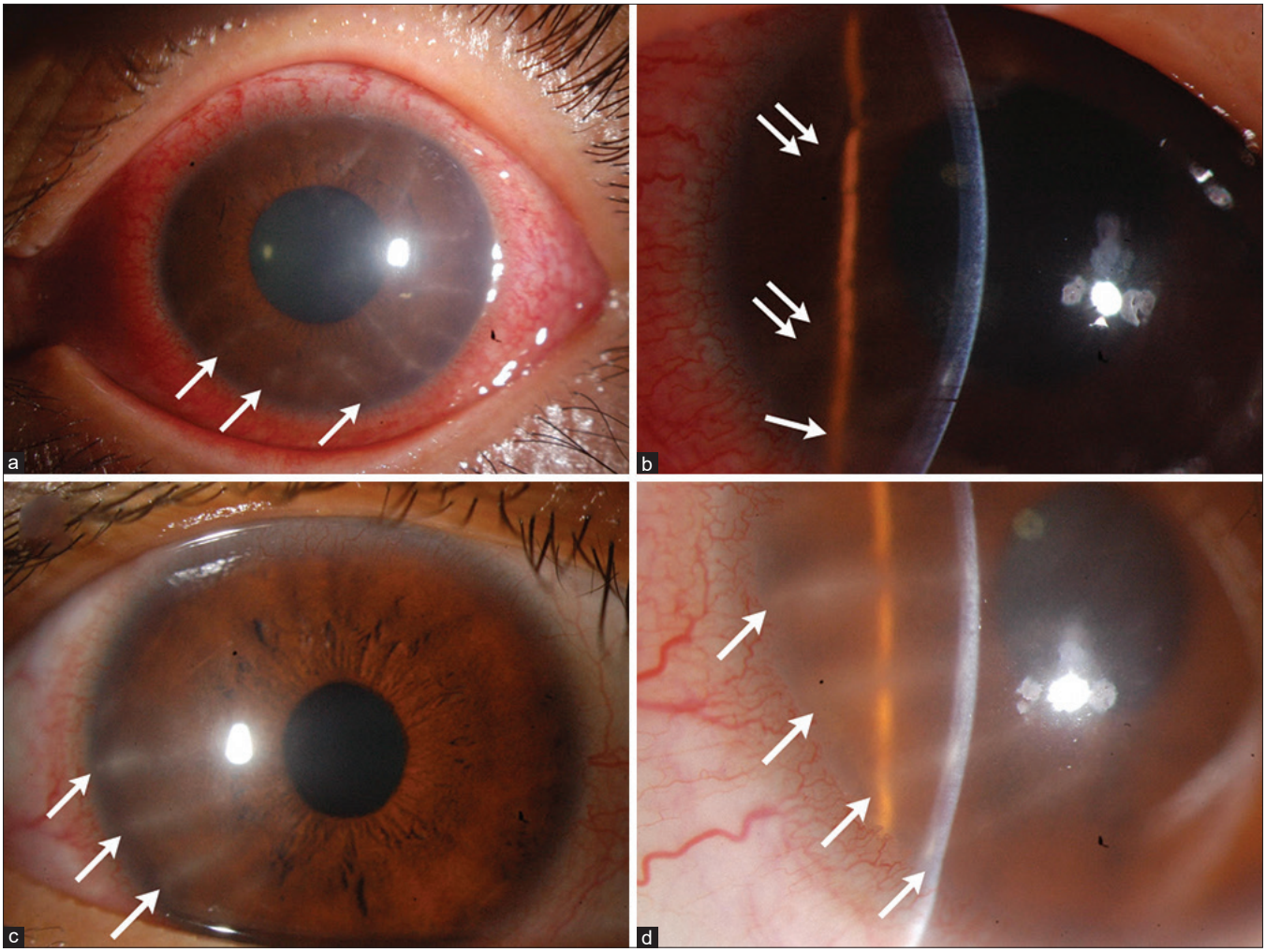

Figure 1: Representative slit-lamp biomicroscopy findings for early-stage *Acanthamoeba* keratitis with radial keratoneuritis (RK; a-d), several areas of keratoneuritis can be observed (Arrows)

Table 1: IVCM findings of 17 eyes (17 patients) diagnosed with early-stage AK with RK

Case No.	Sex/ Age Y)	Amoeba cysts in the epithelium	Amoeba trophozoites in the epithelium	Basal epithelial nerve changes	Amoeba cysts in the stroma	Amoeba trophozoites in the stroma	Honeycomb pattern in the stroma	Spindle-shape materials in the stroma	Thickened stromal nerve with increased density	Thin tortuous stromal nerve	Inflammatory cells in the stroma
1	F/12	+	+			+			+		+
2	F/13				+	+	+		+		+
3	F/14										+
4	F/15	+	+	+			+				+
5	F/15	+	+	+		+			+		+
6	F/17	+	+	+			+				+
7	F/17	+			+		+				+
8	F/22	+		+			+				+
9	F/24	+		+			+	+			+
10	F/27		+	+			+	+			+
11	F/31	+	+	+	+	+			+		+
12	F/34		+							+	+
13	F/24	+	+		+		+	+		+	+
14	M/19	+	+				+				+
15	M/20		+	+			+				+
16	M/22				+	+			+		+
17	M/26							+			+
Total		10/17	10/17	8/17	5/17	5/17	10/17	4/17	5/17	2/17	17/17

Acanthamoeba cysts and trophozoites were observed in the basal epithelial layer in 10 of 17 eyes. Morphological changes in corneal nerves in the basal epithelial layer or Bowman's layer were found in 8 of 17 eyes. In the corneal stromal layer, *Acanthamoeba* cysts were observed in 5 of 17 eyes. Highly reflective, spindle-shaped materials in the stroma were found in 4 of 17 eyes. Activated keratocytes forming a honeycomb pattern were found in 10 of 17 eyes. Thin, tortuous, and fragmented stromal nerves with no change in corneal diameter and were found in 2 of 17 eyes. Five of 17 eyes showed thickened stromal nerves with increased nerve density. IVCM=*In vivo* confocal microscopy, AK=*Acanthamoeba* keratitis, RK=Radial keratoneuritis

Table 2: Pathological IVCM findings in 17 patients with early-stage AK with RK

Corneal Sublayer	Finding	Number of Patients	Sensitivity of *in vivo* confocal microscopy for the detection of AK (%)
Surface epithelium	*Acanthamoeba* cysts	10	58.82
	Acanthamoeba trophozoites	10	58.82
Basal epithelium	Nerve changes	8	47.06
Stroma	*Acanthamoeba* cysts	5	29.41
	Acanthamoeba trophozoites	5	29.41
	Honeycomb patterns	10	58.82
	Spindle-shaped structures	4	23.53
	Thickened nerves with increased density	5	29.41
	Thin, tortuous nerves	2	11.76
	Inflammatory cells	17	100.00

IVCM=*In vivo* confocal microscopy, AK=*Acanthamoeba* keratitis, RK=Radial keratoneuritis

tortuous, fragmented corneal nerves in the basal epithelial layer exhibited increased diameter and density and were mostly distributed parallel to one another [Fig. 2h] after the penetration of Bowman's membrane. Amoeba trophozoites were suspected to be attached to the intrastromal nerve trunks [Fig. 2i]. Table 1 summarizes the above IVCM findings, and Table 2 summarizes the sensitivity of the IVCM findings for the diagnosis of *AK* from corneal scrapes.

Representative case

A 31-year-old woman who wore soft contact lenses for refractive correction in both eyes presented with a complaint of ocular discomfort in the left eye for 2 weeks. The BCVA for the left eye was 20/25. Slit-lamp biomicroscopy showed severe conjunctival infection with numerous swollen corneal nerves that were characteristic of RK emerging from the limbus. IVCM images showed *Acanthamoeba* cysts in the basal epithelial layer [Fig. 3a and b]. A diagnosis of AK with RK was also confirmed by culture positivity for *Acanthamoeba*. A combination of topical polyhexamethylene biguanide, chlorhexidine, propamidine, and clotrimazole was applied hourly. The patient recovered after treatment and remained stable without recurrence.

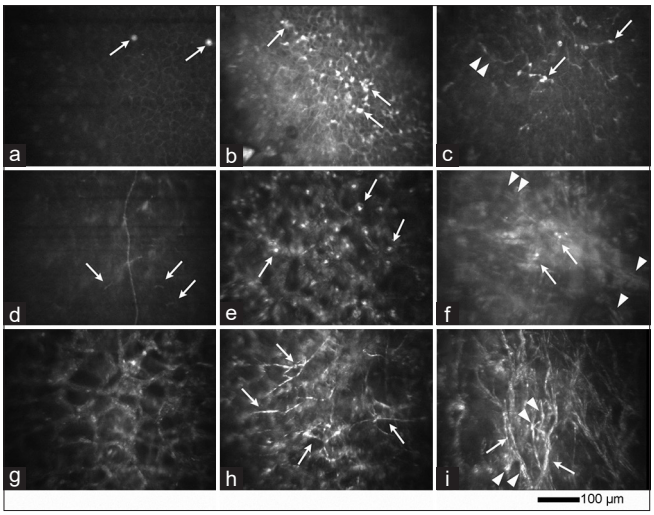

Figure 2: Representative *in vivo* confocal microscopy images for cases of early-stage *Acanthamoeba* keratitis with radial keratoneuritis (a) Typical *Acanthamoeba* cysts in the basal epithelial cells are observed as hyper-reflective, round, bilayered structures with a diameter of 10 to 20 μm (Arrows). (b) Typical *Acanthamoeba* trophozoites in the basal epithelial cells are observed as hyper-reflective, amorphous, irregular, wedge-shaped structures with a diameter of 10 to 20 μm (Arrows). In this image, the trophozoites are not attached to the corneal nerves. (c) Typical *Acanthamoeba* trophozoites in the basal epithelial cells are observed as hyper-reflective, amorphous, irregular, wedge-shaped structures with a diameter of 10 to 20 μm (Arrows). In this image, the trophozoites are attached to the thin and tortuous corneal nerves (Arrowheads) (d) Thin, fragmented corneal nerves in Bowman's layer (Arrows). (e) Numerous *Acanthamoeba* cysts in the corneal stroma are observed as highly reflective, double-walled, round structures with a diameter of 10 to 20 μm (Arrows). (f) Numerous *Acanthamoeba* cysts (Arrows) in the corneal stroma are surrounded by highly reflective, spindle-shaped structures (Arrowheads). (g) In the stroma, highly reflective activated keratocytes form a honeycomb pattern. (h) In the stroma, some eyes show thin, tortuous corneal nerves with a beaded appearance and inconsistent diameters along the same nerve (Arrows). (i) In the stroma, some eyes show engorged, tortuous corneal nerves with increased nerve density and inconsistent reflectivity (Arrows). *Acanthamoeba* trophozoites have attacked the deformed corneal nerves (Arrowheads) Scale bar 100 μm

Serial IVCM images clearly showed spatial changes in the corneal nerves. The basal epithelial layer [Fig. 3a] showed thin, fragmented corneal epithelial nerves with a few highly reflective, coffee bean-shaped particles that were strongly suspected to be *Acanthamoeba* cysts. The deeper layer of corneal nerves near Bowman's layer appeared engorged and tortuous [Fig. 3b]. The diameter of the corneal nerves had increased, and the nerves became more tortuous as deeper layers of the corneal stroma were examined [Fig. 3c]. In the deeper layers, the density of the thickened corneal stromal nerves had also increased. Strongly hyper-reflective, irregular, wedge-shaped structures suspected to be *Acanthamoeba* trophozoites were found along the corneal nerves [Fig. 3d]. The stromal nerves showed a continuous increase in their size, tortuosity, and density with an increase in depth in the middle stroma. Hyper-reflective, irregular, wedge-shaped structures that were strongly suspected to be *Acanthamoeba* trophozoites were still attached to the middle stromal nerves [Fig. 3e and f].

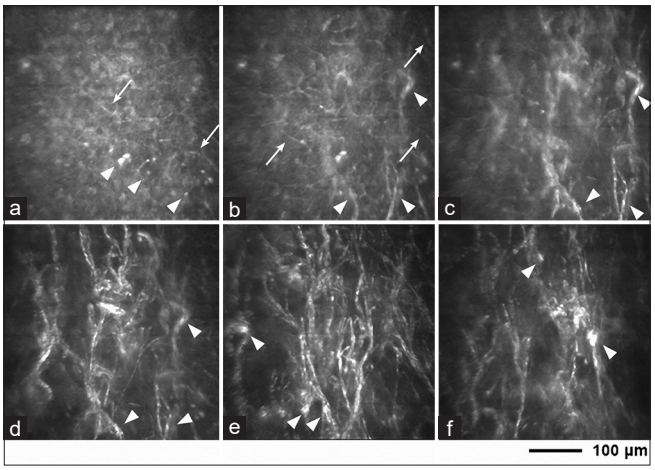

Figure 3: A 31-year-old woman presented with a complaint of ocular discomfort in the left eye for 2 weeks. Slit-lamp biomicroscopy showed severe conjunctival infection with numerous swollen corneal nerves that were characteristic of radial keratoneuritis (RK) emerging from the limbus. A diagnosis of *Acanthamoeba* keratitis (AK) with RK was later confirmed. Serial images obtained through *in vivo* confocal microscopy for early-stage AK with RK in this case. (a) In the basal epithelial layer, a fragmented, thin corneal epithelial nerve (Arrows) can be seen with coffee bean-shaped, hyper-reflective spots that are strongly suspected to be *Acanthamoeba* cysts (Arrowheads). (b) An image obtained at a depth of 4 μm from the basal epithelial layer in (A). The corneal nerve density in the basal epithelial layer has increased (Arrows). Some areas of the corneal nerves appear engorged and tortuous (Arrowheads). The *Acanthamoeba* cysts found in (A) are also observed in this image. (c) An image obtained at a depth of 20 μm from the basal epithelial layer in (A). The stromal nerves show an increase in their size and tortuosity. Amorphous, hyper-reflective, irregular, wedge-shaped structures (Arrowheads) that are strongly suspected to be *Acanthamoeba* trophozoites are attached to a stromal nerve. (d) An image obtained at a depth of 40 μm from the basal epithelial layer in (A). The stromal nerves show a continuous increase in their size, tortuosity, and density. Many amorphous, hyper-reflective, irregular, wedge-shaped structures (Arrowheads) that are strongly suspected to be *Acanthamoeba* trophozoites are attached to the stromal nerve in a pattern that mimics fungal hyphae. (e and f) Serial images of the middle stroma. In alphabetical order, each image has been obtained at a depth of 40 μm from the previous depth. The stromal nerves show a continuous increase in their size, tortuosity, and density. Hyper-reflective, irregular, wedge-shaped structures (Arrowheads) that are strongly suspected to be *Acanthamoeba* trophozoites are still attached to the middle stromal nerves Scale bar, 100 μm

Discussion

To the authors' knowledge, this is the largest case series using IVCM for the detection of early-stage AK with RK and evaluation of spatial changes in the corneal nerves at different depths. Instead of analyzing single cross-sectional images, the nerves on serial images were assessed to account for their three-dimensional structures. The present findings suggested that white light IVCM could identify consistent corneal findings, particularly spatial changes in the corneal nerves, in cases of early-stage AK with RK.

IVCM has been found to exhibit high sensitivity and specificity for AK detection and it can provide information that microbiological tests such as smear and culture cannot.[2-7] There are two major types of IVCM devices in the market:

laser (e.g., HRT; Heidelberg Engineering GmbH) and white light (e.g., Confoscan; Nidek Technologies). Although both HRT and white light IVCM are widely available on the market and widely used in the clinic, they have different features and technical specifications.[8] Confoscan is superior in that it is equipped with an automatic operating mode, which is lacking in HRT. This feature is very convenient for use in the busy clinic and by inexperienced examiners, as it can provide good-quality images within the short examination time and has a short learning curve. Besides, white light IVCM does not have the disadvantage of possible lesion-like artifacts in imaging corneas (e.g., stromal striae and Descemet's membrane folds), which are frequently found in HRT IVCM because of direct microscope application to the cornea.

These two systems have different optical designs and imaging qualities. Confoscan is equipped with a ×40 front lens (Zeiss). Manual, semi-automatic, and automatic operating modes allow the production of back and forth movements during image acquisition. The HRT laser device is equipped with a ×60 front lens (Olympus, Tokyo, Japan) and employs a manual technique for imaging of the entire corneal thickness; a semi-automatic technique covers only the anterior 85 µm. Therefore, the detection of larger or deeper areas may be more difficult with laser IVCM than with white light IVCM, which is especially important for clinical use, as IVCM examination is mostly needed for clinical conditions with corneal endothelial manifestations.

RK is considered an early pathognomonic presentation of AK.[9-12] Several studies, including the present one, have used IVCM to detect *Acanthamoeba* cysts and trophozoites, the honeycomb pattern of active keratocytes, infiltration of inflammatory cells, and highly reflective spindle-shaped structures representative of RK.[6,9] The present study showed that *Acanthamoeba* cysts and trophozoites can be observed in the basal epithelial and stromal layers [Figs. 2 and 3]. *Acanthamoeba* cysts were noted as hyper-reflective, round particles with a diameter of 10 to 20 µm, and *Acanthamoeba* trophozoites were observed to be amorphous, hyper-reflective, bright, irregular, wedge-shaped structures with a diameter of 10 to 20 µm.[13,14] These morphometric findings were typical and consistent with those of a previous study conducted by Kanavi *et al*.[15]

The mechanism underlying the development of RK in eyes with early-stage AK is not fully understood. Most clinicians and researchers believe that RK is caused by an immune reaction to amoebic migration into the corneal stroma and along the corneal stromal nerves.[12,16] It has been surmised that both nerve invasion by trophozoites and the resultant swelling may cause severe pain associated with AK. It has also been suggested that *Acanthamoeba* trophozoites track along and feed on the corneal nerves.[6] A study by Kobayashi *et al*. used the HRT laser IVCM system to detect corneal pathological changes in 13 eyes with RK[9] and found that *Acanthamoeba* cysts existed almost exclusively in the basal epithelial cell layer. No stromal or nerve infiltration by *Acanthamoeba* cysts and trophozoites was found and it was concluded that RK may be mediated by inflammatory molecules such as cytokines that are released by the *Acanthamoeba* localized in the corneal epithelium. Immune or inflammatory mediators may diffuse through the corneal stroma into the corneal nerves or affect corneal nerve endings in the epithelial cell layer. In the present study, seven of 17 eyes exhibited *Acanthamoeba* cysts or trophozoites in the corneal stroma, with some of these cysts/trophozoites attached to the corneal nerves [Fig. 2e, f and i]. This finding suggests that *Acanthamoeba* cysts and trophozoites have already penetrated the corneal stroma in the early stages of AK with RK. The development of RK may be partially attributed to a direct attack of the corneal nerves by *Acanthamoeba*.

Alterations in the morphology and density of the corneal nerves in early-stage, late-stage, and cured AK have been found in studies using laser IVCM.[17-19] In these studies, the corneal nerve density and length were found to have decreased in the acute stage of AK, followed by regeneration in later stages. In contrast, a study by Kanavi *et al*. found irregular, thickened corneal nerves with a beaded appearance on white light IVCM images.[5] The present study showed various patterns of corneal nerve changes in cases of early-stage AK with RK. While some eyes demonstrated thin, tortuous, fragmented nerves in the basal epithelial layer [Fig. 2c], Bowman's layer [Fig. 2d], and stroma [Fig. 2h] without significant alternations in the morphology or diameter at different corneal depths, some demonstrated thin, fragmented corneal nerves in the basal epithelial layer [Fig. 3a] that became thickened, tortuous, and denser after penetrating the corneal stroma [Fig. 3b-d]. Several bright, irregular, wedge-shaped structures suspected to be *Acanthamoeba* trophozoites were found attached to the corneal stromal nerves [Fig. 3d-f]. The serial images in Fig. 3 suggest that *Acanthamoeba* gained access to the corneal stroma by following the corneal epithelial nerves that penetrated Bowman's layer to enter the stroma, thus causing the nerves to undergo significant morphological changes. To the best of the authors' knowledge, such a presentation has not been previously reported. There are two main reasons for this observation not having been reported by other studies. First, most of the other studies used HRT instead of Confoscan as the imaging tool,[13,20-22] and the different optical designs may have yielded different results. Because images of the transformed corneal nerves obtained with Confoscan can mimic fungal hyphae [Figs. 2h, i and 3c, d], careful evaluation of the three-dimensional structure and differentiation of deformed corneal nerves from fungal hyphae are necessary. Second, the interpretation of IVCM images is mostly dependent on the morphological presentation, and it cannot be contested with certainty that the bright linear areas presented in Fig. 3 were corneal nerves and not stromal tracts created during the migration of trophozoites into the stroma and along the corneal nerves. The findings of a study by Kiderlen *et al*., where a migratory pathway of the "brain-eating" amoeba *Naegleria fowleri* was found, support this possibility. It was theorized that amoebae can invade the olfactory epithelium by traveling along the olfactory nerves via holes in the cribriform plate and reach the olfactory bulb to cause severe necrotizing meningoencephalitis.[23] Therefore, further studies are required to confirm corneal nerve changes and the migration pathway of *Acanthamoeba* from the ocular surface into the corneal stroma in cases of AK.

This study has some limitations. First, white light IVCM with an automatic capture technique was used to evaluate the included patients. The disadvantage of this technique is that it is difficult to maintain the patient's head position. This may explain why *Acanthamoeba* cysts or trophozoites were detected in neither the epithelium nor the stroma in cases 3 and 17.

Second, even though this was the largest case series using white light IVCM to evaluate early-stage RK with AK, the sample size may have been small, and further larger studies using the manual operating mode of the white light IVCM device are required to confirm these results.

Conclusion

In conclusion, the present findings suggest that white light IVCM provides useful information for the diagnosis of early-stage AK with RK. It can detect the presence of *Acanthamoeba* trophozoites and cysts, the honeycomb pattern of activated keratocytes, highly reflective spindle-shaped structures, and changes in the corneal nerves that are characteristic of this disease. Pathological corneal nerves undergo spatial changes at different depths, and careful interpretation of this finding is important for an accurate diagnosis of AK with RK.

Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form, the patient(s) has/have given his/her/their consent for his/her/their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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