

Acridine Orange Staining for Rapid Diagnosis of *Acanthamoeba* Keratitis

Tae-Won Hahn,* Terrence P. O'Brien,[†] Woo-Jin Sah* and Jae-Ho Kim*

*Department of Ophthalmology, Catholic University Medical College, Kangnam St. Mary's Hospital, Seoul 137-040, South Korea; [†]Ocular Microbiology Laboratory, The Wilmer Institute, Johns Hopkins Hospital, Baltimore, MD, USA

Abstract: *Acanthamoeba* keratitis is uncommon, but one of the most severe infectious diseases of the cornea. Delayed diagnosis or misdiagnosis as bacterial or herpes simplex keratitis leads to extensive corneal inflammation and profound visual loss. Therefore, accurate and rapid diagnosis of *Acanthamoeba* keratitis is essential for successful treatment and good prognosis. We evaluated the usefulness of acridine orange staining from corneal scrapings and contact lens solutions for the rapid diagnosis of four consecutive cases of *Acanthamoeba* keratitis. Gram stain and culture on nonnutrient agar plates with *Escherichia coli* overlay were also made. Corneal scrapings stained with acridine orange revealed yellow-to-orange polygonal, cystic structures consistent with the appearance of *Acanthamoeba* among inflammatory cells and the corneal epithelial cells. The contact lens case solutions of two patients also showed numerous cysts with double wall. Some organisms from the third patient were identified as *Acanthamoeba castellanii* and others as *Acanthamoeba lugdunensis*. Based on the acridine orange staining results in four cases of *Acanthamoeba* keratitis, this stain is recommended as a simple and reliable method for the rapid diagnosis of this disease. **Jpn J Ophthalmol 1998;42:108-114** © 1998 Japanese Ophthalmological Society

Key Words: *Acanthamoeba* keratitis, acridine orange stain, contact lens.

Introduction

Acanthamoeba is now a well-known causative organism of chronic infectious keratitis, especially among contact lens users. Since the first report of *Acanthamoeba* keratitis in 1975,¹ several hundred cases have been reported, and this uncommon disease has attracted ophthalmologists' attention because of the difficulty in early diagnosis and successful medical treatment.

Acanthamoeba keratitis can be diagnosed by several typical clinical features and a routine microbiologic investigation. However, the presenting clinical findings of *Acanthamoeba* keratitis are sometimes variable, and laboratory diagnosis is possible only if an experienced observer examines the smear and

culture specimens with an awareness of this infection. Early diagnosis of *Acanthamoeba* keratitis is especially important for successful medical cure. This significant point has been stressed by several ophthalmologists.²⁻⁴

Acanthamoeba cysts, or trophozoites, can be stained with conventional staining methods, but there is a possibility that the amoeba will be stained poorly or be mistaken for mononuclear cells or degenerated epithelium. Special stainings with calcofluor white or indirect fluorescent antibody have been suggested as rapid and reliable diagnostic methods for *Acanthamoeba* keratitis, but such procedures are complicated compared to acridine orange staining.^{5,6}

Acridine orange, a fluorochromatic dye, has been used to stain bacteria or fungi from infectious keratitis.^{7,8} With an acidic buffer, acridine orange shows bacteria in orange fluorescence, and human cells or other background materials in green-yellow fluorescence.⁹ This remarkable differential staining effect of acridine orange is superior to Gram stain, and enables an observer to identify the infectious organism

Received: January 22, 1997

Address correspondence and reprint requests to: Tae-Won HAHN, MD, Department of Ophthalmology, Catholic University Medical College, Kangnam St. Mary's Hospital, 505 Banpo-dong, Seocho-ku, Seoul 137-040, Korea
This paper was presented at the World Congress on the Cornea IV (1996) in Orlando, FL, USA.

easily and quickly. We prospectively used this stain in four consecutive cases as an initial diagnostic method, together with Gram stain and culture, and evaluated the usefulness of acridine orange for the rapid diagnosis of *Acanthamoeba* keratitis.

Patients and Methods

Patients

Between August 1995 and April 1996, *Acanthamoeba* keratitis was diagnosed and confirmed in four keratitis patients by corneal scrapings and culture at the Department of Ophthalmology of the Kangnam St. Mary's Hospital, Seoul. Among the four patients, three were daily-wear soft contact lens users and one did not wear contact lenses. Because of the suspicion of *Acanthamoeba* keratitis based on clinical symptoms and signs, smears of corneal scrapings were made on two sterilized glass slides for acridine orange and Gram stains. Culture was done on nonnutrient agar plates with *Escherichia coli* in all four patients.

Acridine Orange Stain

Acridine orange staining procedures are simple: Sterilized slide is fixed with 95% methanol for 2 minutes. Excess methanol is drained and slide is allowed to air-dry. Slide is flooded with acridine orange stain (Vacutainer, Becton Dickinson, Mountain View, CA, USA) for 2 minutes, rinsed thoroughly with tap water, and allowed to air-dry. Smears are examined at $\times 400$ and $\times 1000$ magnification with a Zeiss fluorescence microscope.

Cultured trophozoites from agar plates were collected and stained with acridine orange to compare their staining characteristics with the cyst form.

Results

Results of the microbiologic investigations in the four cases are summarized in Table 1. Corneal scrapings stained with acridine orange revealed yellow-

to-orange polygonal, cystic structures consistent with the appearance of *Acanthamoeba* among inflammatory cells and corneal epithelial cells. The acridine orange staining characteristics of *Acanthamoeba* differed according to stage. Most commonly observed *Acanthamoeba* from corneal scraping specimens or contact lens case solutions were in the encysted form, and showed a typical double wall with yellow fluorescence under fluorescence microscopy. Figures 1 and 2 show polygonal *Acanthamoeba* cysts from the corneal scrapings of cases 2, 3, and 4. In the contact lens case solution of case 1, numerous encysted *Acanthamoeba* were also found (Figure 3). Other *Acanthamoeba* cysts observed in the same slide of case 2 had a small, round configuration and bright-orange fluorescence (Figure 4). These cysts seemed to be in the active stage, just completing division. An *Acanthamoeba* cyst from case 1 had polygonal shape with a single wall (Figure 5). It was also thought to be in the active stage.

The trophozoite form was rarely seen in specimens of corneal scrapings and contact lens solutions. In order to observe the staining characteristics of trophozoites with acridine orange, we collected trophozoites from axenic culture and stained them with acridine orange. Different from the encysted ameba, the stained trophozoites appeared with uniform bright-orange fluorescence (Figure 6).

Case Reports

Case 1

A 23-year-old woman had used daily-wear soft contact lenses intermittently for several years. After wearing a contact lens that was unused for several months and stored in a lens case with saline, she developed foreign-body sensation, photophobia, ocular pain, and tearing in her right eye. Her corrected visual acuity was 20/200 in the eye. Slit-lamp examination revealed a mild conjunctival follicular reaction and 5-mm oval-shaped stromal infiltrate in the superficial stroma. Multiple epithelial defects existed

Table 1. Microbiologic Data

Case No.	Sex/Age	Corneal Scrapings			CL Case Solution			Species Identification (by Morphology, Riboprints, and RFLPs)
		AO	Gram Stain	Culture	AO	Gram Stain	Culture	
1	F/23	+	+	+	+	+	+	<i>Acanthamoeba lugdunensis</i>
2	M/17	+	+	+	-	-	+	<i>A. lugdunensis</i>
3	F/16	+	+	+	-	-	-	<i>A. castellanni</i>
4	M/32	+	+	+		Not Available		<i>A. lugdunensis</i>

AO = acridine orange.

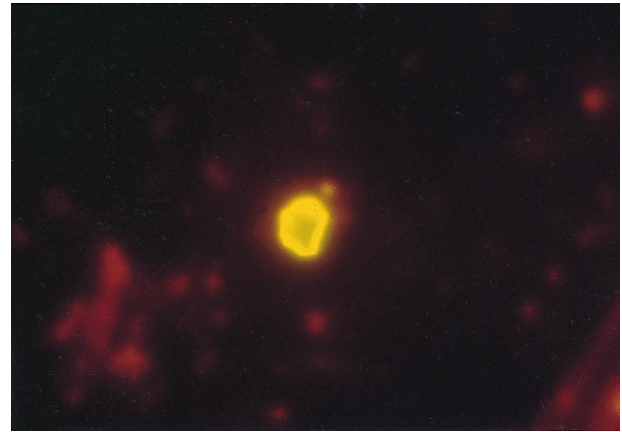
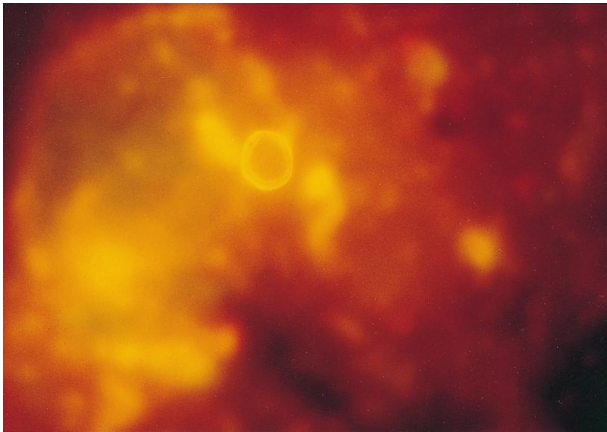


Figure 1-2. Yellow-to-orange fluorescent polygonal amebic cyst with double wall detected in corneal scraping specimens from cases 2, 3, and 4 (Original magnification $\times 1000$).

over the stromal infiltrate region. There were several keratic precipitates, and perineural infiltrates along several corneal nerves to the limbus were noted (Figure 7). Corneal scraping specimens were obtained for Gram and acridine orange stains and culture on nonnutrient agar with *E. coli* overlay. The contact lens case solution and the patient's used saline were examined with acridine orange staining. *Acanthamoeba* cysts were found in both the corneal scraping specimens and the contact lens case solution. The organism was identified as *A. lugdunensis* by its morphology, riboprint, and restriction fragment length polymorphisms of mitochondrial DNA. Treatment with topical polyhexamethylene biguanide (PHMB, 0.02%), neomycin-polymyxin bacitracin ointment, and systemic itraconazole was initiated. Six months after treatment, a faint su-

perficial stromal scar remained and corrected visual acuity improved to 20/25 in the affected eye.

Case 2

A 17-year-old man visited a private practitioner because of ocular discomfort after wearing daily-wear soft contact lenses. Herpes simplex keratitis was diagnosed and he was treated for 2 weeks. At the first examination of the affected eye, there was a round central epithelial defect and dense stromal infiltrates. Numerous keratic precipitates existed and the anterior chamber had a mild degree of cells and flare (Figure 8). Corneal scrapings obtained with suspicion of *Acanthamoeba* keratitis showed active and encysted amebic cysts after Gram and acridine

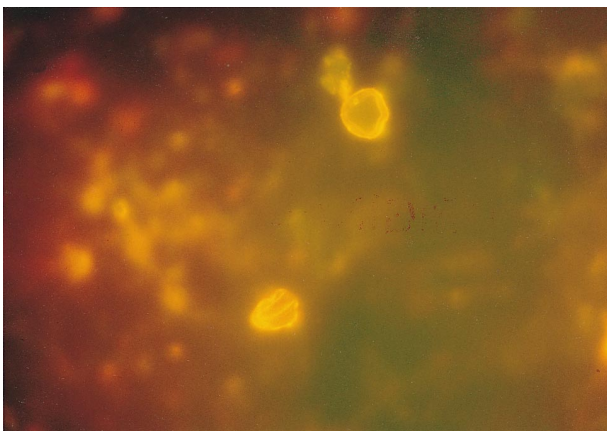


Figure 3. Polygonal cysts from contact lens case solution fluorescent yellow-to-orange in their wrinkled walls (Original magnification $\times 1000$).

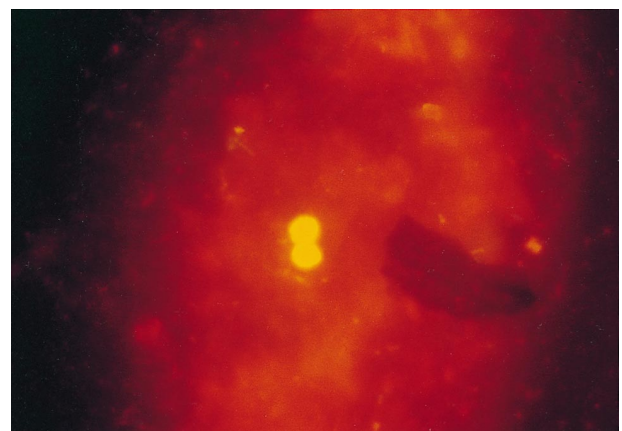


Figure 4. Active cysts just completing division show round configuration and bright orange fluorescence (Original magnification $\times 400$).

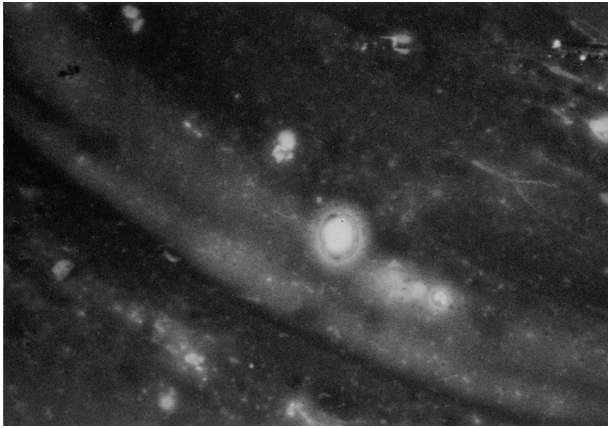


Figure 5. Cyst found in corneal scrapings from case 1 (Original magnification $\times 400$).

orange stain. Ameba were not found in the smear and culture of the contact lens case solution or the patient's used saline. *A. lugdunensis* grew from the culture of corneal scrapings. Treatment was changed to topical PHMB (0.02%), hexamidine (0.1%, Desomedine, Chauvin-Blache, France), and systemic itraconazole. After 8 months of medical treatment, the round central stromal opacity with stromal thinning remained. Instillation of topical PHMB and fluorometholone are still continuing, with tapering.

Case 3

A 16-year-old girl was referred because of severe central suppurative corneal ulcer and nodular scleritis in her left eye (Figures 9 and 10). She had been treated for bacterial keratitis for 3 months since she had developed ocular pain and tearing in her left eye

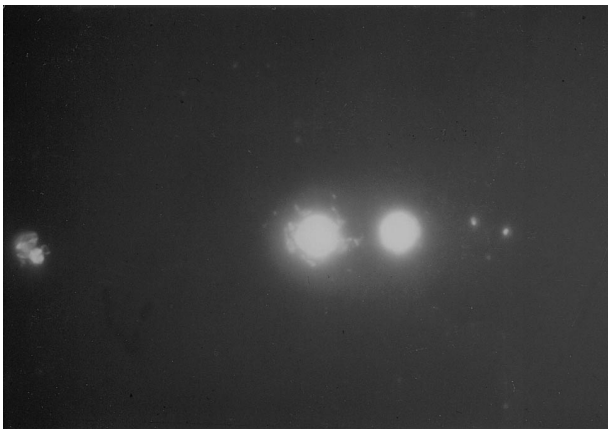


Figure 6. Trophozoites from axenic culture show bright orange fluorescence and typical acantopodia (Original magnification $\times 1000$).

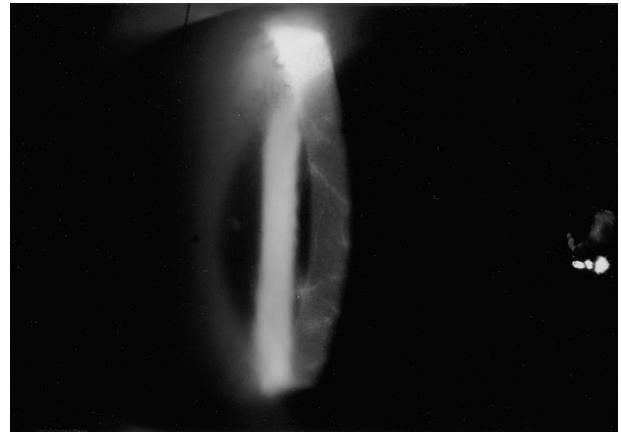


Figure 7. Case 1: Pathognomonic sign of *Acanthamoeba* keratitis, perineural infiltrates along corneal nerves visible from lesion to limbus.

after wearing daily-wear soft contact lenses. Aggravation of corneal inflammation despite strong antibiotic therapy, nodular scleritis, and contact lens-related infection suggested the possibility of *Acanthamoeba* infection. Corneal scrapings stained with acridine orange and Gram showed yellow-to-orange fluorescent amebic cysts with double wall. The smear and culture of her contact lens case solution was on nonnutrient agar, but the bacteria identified on the scraping specimens did not grow on routine culture plates. Combination therapy with PHMB (0.02%), hexamidine (0.1%), and systemic itraconazole was started, and topical ofloxacin was also instilled for possible mixed infection and prevention of secondary bacterial infection. Despite treatment, central corneal perforation occurred 3 weeks later. Penetrating keratoplasty was performed

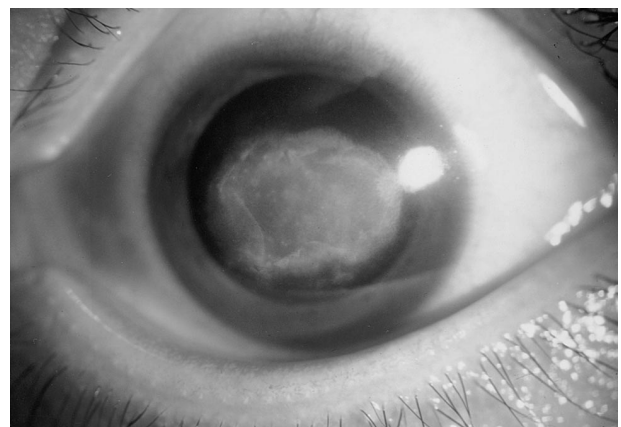
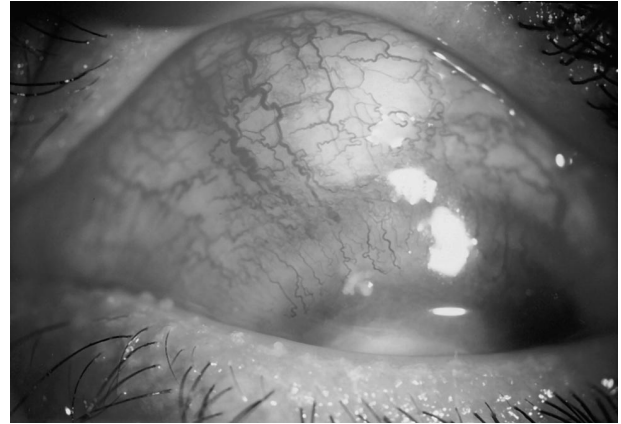
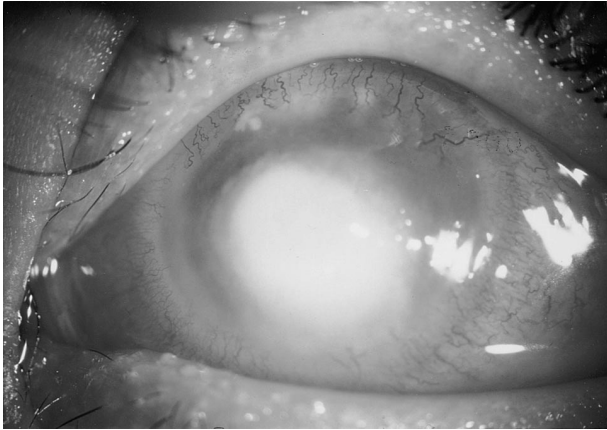


Figure 8. Case 2: Round epithelial defect and dense stromal infiltrates develop at central cornea.



Figures 9–10. Case 3: Marked suppurative corneal ulcer occurs at central cornea; nodular scleritis also seen at 12 o'clock position.

and antiamebic therapy continued. Up to 8 months after keratoplasty, there is no sign of recurrence on the donor cornea.

Case 4

A 32-year-old man had been treated for fungal keratitis for 2 months at two different eye clinics after suffering a spontaneous onset of mild ocular pain, redness, and a decrease of visual acuity in his left eye. He did not have a history of ocular trauma and had not worn contact lenses. Slit-lamp examination showed moderate conjunctival and ciliary injection, 8-mm-sized central epithelial defects with round stromal infiltrate and hypopyon. Pupil and iris structures were barely visible (Figure 11). Corneal scrapings stained with acridine orange and Gram demonstrated amebic cysts. The organism was identified as *A. lugdenensis*. PHMB, neomycin ointment, 2% homatropine, and systemic itraconazole were prescribed. Topical prednisolone acetate was added with the resolution of stromal infiltrates and the epithelial defect. After 3 months of treatment, keratoplasty was performed to correct central corneal perforation.

Discussion

The diagnosis of *Acanthamoeba* keratitis is difficult because the clinical features of this disease are variable and corneal scrapings may not include the organism if examined too early in the course of the disease. Therefore, clinical awareness after chronic corneal ulcer is unresponsive to antibiotic therapy seems to be the first, most important consideration for the diagnosis of *Acanthamoeba* keratitis.

Microscopic examination of the slide by an experienced observer is highly recommended. Scrapings of the cornea are stained by Giemsa, trichrome, Wright's, or Gram stain, but amebic cysts can be mistaken for mononuclear cells or degenerated epithelium. Calcofluor white stain was reported to be a fast and reliable method to detect amebic cysts in corneal smears and biopsy specimens.⁶ It stains amebic cysts bright apple-green, and Evens blue counterstain makes background materials or trophozoites red-brown. Indirect fluorescent antibody staining was also suggested for the diagnosis of *Acanthamoeba* keratitis, but this procedure is more complicated and a special antibody is required.⁵

Compared to other staining methods, acridine orange stain is simpler and faster. It requires only one

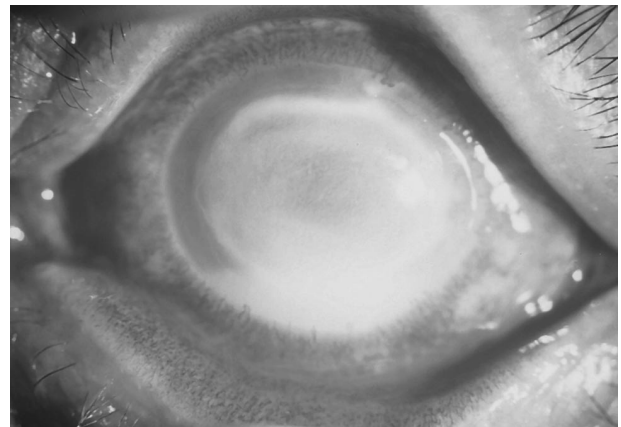


Figure 11. Case 4: Large central epithelial defect with dense stromal infiltrates involves almost entire cornea; hypopyon noted.

staining solution and can be done within a few minutes on outpatient clinical basis.

Prominent color contrast is another advantage of acridine orange stain. With an acidic buffer, acridine orange results in orange fluorescence of the bacteria and green-yellow fluorescence of the human cells and background materials.⁸ Because of these staining characteristics, it has been used to detect the causative microorganisms in infectious keratitis and was reported to be more sensitive than Gram stain.^{7,9,10} We used acridine orange stain (Vacutainer, Becton Dickinson, Mountain View, CA, USA) in 0.01% aqueous solution (pH 4). Methanol or acetone/alcohol mix is recommended for fixation by the company. Kronvall et al.⁸ also evaluated various fixation methods for bacteria in acridine orange staining, such as air-drying, methanol, chloroform, 10% formaldehyde, or ethyl ether: ethanol 1:1. No differences in results were seen among these procedures.

With acridine orange stain, amebic cysts from corneal scrapings or contact lens solutions showed several staining features. Encysted *Acanthamoeba* showed typical polygonal configuration with double wall and yellow-to-orange fluorescence. However, structures inside the cysts were not stained. It seems that acridine orange dye binds to the mucopolysaccharide component of the cyst wall, but does not penetrate its double wall. On the other hand, active cysts have a small, round shape and uniform yellow-to-orange fluorescence. Tissue autofluorescence can be seen under fluorescence microscopy, but it is the morphologic characteristics and orange-to-yellow fluorescence of the cysts that help the observer identify the organisms easily. As with acridine orange, Gram stain also shows a similar configuration of amebic cysts,

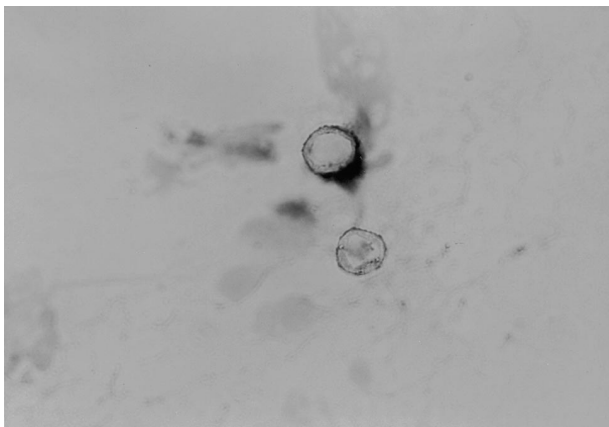


Figure 12. Gram stain shows typical feature of encysted amebae with double wall (Original magnification $\times 1000$).

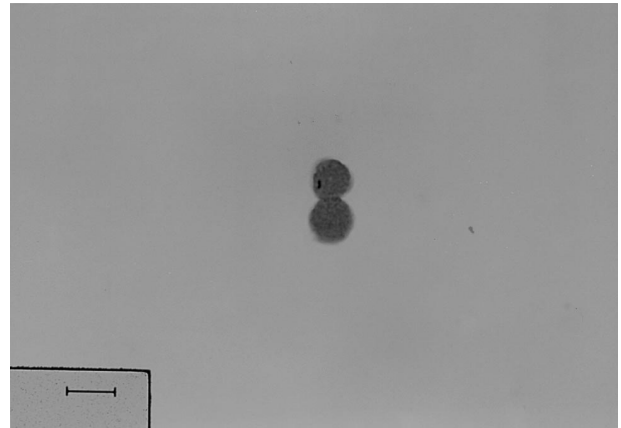


Figure 13. Gram stain over acridine orange-stained smear specimen shows active-stage cysts that are dividing (Original magnification $\times 1000$).

such as polygonal shape with double wall. The double wall is discernible because of violet-blue color and active cysts usually show pink staining (Figures 12 and 13).

It is hard to identify the trophozoite form in corneal scrapings because of its variable morphology. With acridine orange stain, trophozoites from axenic culture differ from the encysted ameba and can be identified by their uniform bright-orange fluorescence. We found a trophozoite in the Gram-stained corneal scrapings from case 4. Confirmation as a trophozoite was difficult under light microscopy because of its atypical shape and indistinct border, but

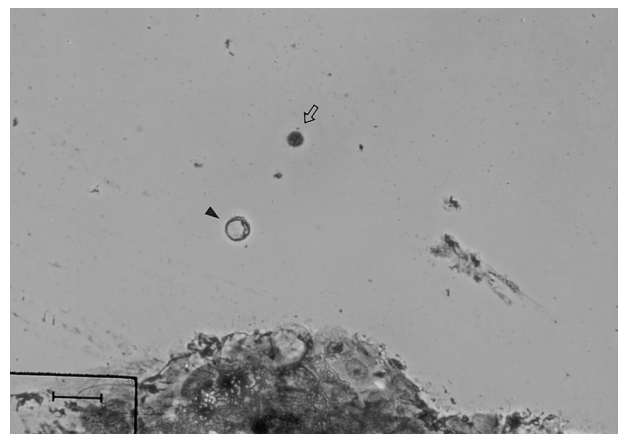


Figure 14. Phase contrast microscopic finding of trophozoite (arrow), which is difficult to find under light microscopy due to faint, multiform cytoplasm. Encysted ameba shown together (arrowhead) (Original magnification $\times 400$).

phase contrast microscopy revealed trophozoite configuration (Figure 14).

The prospective use of acridine orange in four cases of *Acanthamoeba* keratitis proved that acridine orange stain is as effective as Gram stain to obtain a positive result from corneal scraping specimens. It was easier for the examiner to identify the amebic cysts from the specimens stained with acridine orange compared to Gram stain specimens. Although access to fluorescence microscopy is a prerequisite for assessing the results of acridine orange staining, considering the simple, fast staining procedure and the easy identification of the microorganisms, we believe that, together with culture, acridine stain is a useful diagnostic tool for the rapid diagnosis of keratitis.

References

1. Jones DB, Visvesvara GS, Robinson NM. *Acanthamoeba polyphaga* keratitis and *Acanthamoeba* uveitis associated with fetal meningoencephalitis. *Trans Ophthalmol Soc UK* 1975;95:221–32.
2. Berger ST, Mondino BJ, Hoft RH, et al. Successful medical management of *Acanthamoeba* keratitis. *Am J Ophthalmol* 1990;110:395–403.
3. Ficker L. *Acanthamoeba* keratitis—the quest for a better prognosis. *Eye* 1988;2(Suppl):S37–45.
4. Moore MB, McCulley JP. *Acanthamoeba* keratitis associated with contact lens: six consecutive cases of successful management. *Br J Ophthalmol*. 1989;73:271–5.
5. Epstein RJ, Wilson LA, Visvesvara GS, et al. Rapid diagnosis of *Acanthamoeba* keratitis from corneal scrapings using indirect fluorescent antibody staining. *Arch Ophthalmol* 1986; 104:1318–21.
6. Wilhelmus KR, Osato MS, Font RL, et al. Rapid diagnosis of *Acanthamoeba* keratitis using calcofluor white. *Arch Ophthalmol* 1986;104:1309–12.
7. Gordon LR, Rodniti J, Brisner JH, Genvert GI. Acridine orange and Gram stains in infectious keratitis. *Cornea* 1990; 9:122–4.
8. Kronvall G, Myhre E. Differential staining of bacteria in clinical specimens using acridine orange buffered at low pH. *Acta Pathol Microbiol Scand* 1977;85:249–54.
9. Kanungo R, Srinivasan R, Sambasiva Rao R. Acridine orange staining in early diagnosis of mycotic keratitis. *Acta Ophthalmol* 1991;69:750–3.
10. Lauer BA, Reller LB, Mirrett S. Comparison of acridine orange and Gram stains for detection of microorganisms in cerebrospinal fluid and other clinical specimens. *J Clin Microbiol* 1981;144:201.