Morphological Features of *Acanthamoeba* Causing Keratitis Contaminated from Contact Lens Cases

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Objective: *To study the morphological characteristics of genus Acanthamoeba which is an opportunistic organism associated with wearing contact lenses that the biofilm phenomenon in contact lens cases contained Acanthamoeba causing keratitis by conventional culture technique.*
Material and Method: *A total of 150 contact lens cases were biofilm scraped in March till September 2007, at an institution in Nakhornpathom Province, Thailand. The 'gold standard' culture technique was used for the excystation growth development observation. Cysts of Acanthamoeba spp. contained 50 microlitres of Escherichia coli and contact lens solution were incubated and observed for the presence of cysts and/or trophozoites for 12 days. An infected slide was stained with giemsa solution and other non-stained and non-fixed slides were carried out for morphological characteristics study by different microscopes.*
Results: *The prevalence of Acanthamoeba spp. in scraping of contact lens cases was 6.7% (10/150). These Acanthamoeba isolates at temperature around 37^{O}C were consisted of all three groups, which in summary; the average diameter of cysts in Astronyxids (group I) was relatively large. They were $\geq$ 18 micrometers, while those of Polyphagids (group II) and Culbertsonids (group III) were $\leq$ 18 micron. The typical morphology of Acanthamoeba trophozoites moving freely in water were recognized by the presence of lobopodium and acanthopodia within 12 observed days. The average size of Acanthamoeba trophozoites was in the range of 12-45 micron. Three different images of cyst were feature studied.*
Conclusion: *Three Acanthamoeba groups by biofilm scraping from contact lens cases should be differentiated. Morphological characteristics cysts and trophozoites should be confirmed. In addition, to improve contact lens wearer education, compliance with contact lens cases, hygiene recommendations and regular disposal of contact lens cases might help to solve contact lens cases.*

Keywords: *Acanthamoeba keratitis, Contact lens cases, Morphological features*

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Acanthamoeba is a genus of free-living amoebae, of which some species could cause opportunistic infections in human with a variety of clinical symptoms including a sight-threatening eye disease known as *Acanthamoeba* keratitis (AK)[1]. The disease was first described in the United States in 1973[2] and then several hundreds of cases were reported worldwide. Contact lens wearers are most at risk from infection and account for 62-71% of AK cases[3]. Biofilms are known to play an important role in the pathogenesis of AK in wearers of contact lenses[4,5]. Roongruangchai and Supadirekkul[6] reported 2.4% (2/87) prevalence of *Acanthamoeba* spp. in contact lens cases. Kosrirukvongs et al[7] reported 6 cases of AK at Siriraj Hospital from January to October 1999 and they were treated with chlorhexidine. Jongwutiwes and colleagues[8] identified two keratitis patients at Chulalongkorn Memorial Hospital on June 15, 1988 and March 12, 1990. Clinical diagnosis is based

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on the presence of keratitis with severe pain and photophobia, ring like stromal infiltrates, radial keratoneuritis, and sometimes pseudodendriform epithelial lesions. In 2001, Savitri et al[9] described a simple procedure of Immunoperoxidase (IP) technique, using indigenously raised antibody, to screen corneal scrapings for *Acanthamoeba* cysts and trophozoites. The validity of the IP test in detection of *Acanthamoeba* cysts and trophozoites was measured by sensitivity, specificity, positive predictive value and negative predictive value in comparison with calcofluor white staining and culture. The IP test had a sensitivity of 100%, a specificity of 94%, and the culture had a sensitivity of 83%, a specificity of 100%. As Lek-Uthai et al[10] studied a method employing loop-mediated isothermal amplification (LAMP) of 18S ribosomal RNA gene to detect *Acanthamoeba* in contact lens cases, they detected visual inspection of turbidity a minimum of 10 pg of *Acanthamoeba* DNA, this technique has more cost and complicated. In 2003, Pasricha et al[11] compared the results of PCR with culture and smear. Based on culture results as the "gold standard" the sensitivity of PCR was the same as that of the smear (87.5%). *Acanthamoeba* grown rapidly on 1.5% non-nutrient agar layered with *Escherichia coli* (*E. coli*) nutrition. The characters of cysts look like a crystal. Stained cysts and trophozoites for species identification or subculture on fresh overlaid *E. coli* culture plates were observed. So agar culture[12] is the mainstay for laboratory detection of *Acanthamoeba*. Culture technique that requires familiarity with the morphology of cysts and trophozoites of *Acanthamoeba*, and it may take several days. The morphological characteristics of growth development study, the proper and cheapest culture method were well characterized.

Material and Method

Since March till October 2007, 150 students from Nakhornpathom Province who used contact lens have been written consent form which was approved by Mahidol University (Ethical Clearance no. MU 2007-038) as appropriate and contact lens cases were collected, each of them was sealed in plastic bag during transportation to the laboratory. The contact lens cases were opened under aseptic conditions then a drop of the amoeba saline was dropped into each contact lens case. A sterile cotton bud was scraped over the internal surface of the contact lens cases. The solution in the contact lens cases was sediment by centrifugation at 2,000 x g, for 5 min. The supernatant was discarded. The pellets were obtained and then subjected to culture. The 50 microlitres of cysts of *Acanthamoeba spp* were dropped onto the contact lens cases containing contact lens solution and 50 microlitres of *E. coli* then they were incubated at room temperature and observed for the presence of cysts and/or trophozoites for 12 days. Slides were identified with giemsa staining, and non-stained and non-fixed slides were also performed for morphological characteristics and excystation study.

The cultivation of Acanthamoeba on non-nutrient agar

The scraped samples from contact lens cases were identified by using cultivation technique. Approximately 5 mL aliquots of late log phase culture of *E. coli* were poured onto non-nutrient agar plates containing 1% (w/v) Oxoid No. 1 agar in Page's amoeba saline (PAS) and left for 5 min. Excess culture fluid was removed and plates were left to dry before being inoculated with an environmental sample. Pellets from the centrifuged solution in the contact lens cases were aseptically dropped onto agar plates which then were incubated at 36.5°C and observed under an inverted microscope for 12 days for the presence of cysts and/or trophozoites. Trophozoites were usually found growing away from the area of bacterial inoculation. Bacterial culture was grown in TSB medium, washed with PAS and inactivated at 60°C for 15 min before use. Turbidity of culture suspension was adjusted to be equal to 0.5 McFarland standards (approximately 10^8 CFU/mL).

Atomic Force Microscopy scanned of non-stained and non-fixed Acanthamoeba cysts

Atomic Force Microscopy (AFM) has been used to visualize nano-scale structure of cellular components. The single non-stained and non-fixed cyst on a thin smear slide was air-dried and scanned. AFM, the Dimension 3100 model with a Nanoscope IIIa controller (Veeco, Santa Barbara, CA) was used. The probes used for imaging were 200 μm long by 20 μm wide single-beam shaped cantilevers (Model ORC8, Veeco) with tip radius of curvature less than 20 nm and spring constant of 0.05 N/m. A piece of Eppendorf CELLocate coverslip with 55 micron grid size (Eppendorf AG, Hamburg, Germany) was glued to the back of the smeared glass slide to locate the scanned cells. Both height and deflection image was captured at a resolution of 512 x 512 and scan rate of 0.5 to 2Hz depending on the scan scale which can range

from tens of micron to hundreds of nanometers. After AFM image was taken, the glass slide was observed under an inverted microscope (Leica, Germany) equipped with an oil immersion 100 x objective len.

Results

The prevalence of Acanthamoeba spp. in contact lens wearers

From 150 contact lens cases, *Acanthamoeba spp* was found in 10 samples, The prevalence was 6.7% (10/150). Cysts of the isolated amoebae were studied morphologically and divided into 3 different subgenera according to the characteristics[13].

The cysts of *Acanthamoeba* spp. from the cultivation were observed for growing development of excystation. They were dropped onto the contact lens cases containing contact lens solution and *E. coli*, and then incubated with temperature record, ranging from 36.8-37.2°C and observed for the presence of cysts and/or trophozoites for 12 days. Three different images of cyst, a non-stained slide were imaged from optical microscope and AFM. The results were shown in Table 1. The fixed-trophozoite could not be scanned by AFM because the cantilever to find out was not suitable for the cell surface.

Morphological characteristics

Cysts

Based on the characteristics described, these *Acanthamoeba* on non-nutrient agar (Fig.1) and isolates from contact lens cases at day 5 (Fig. 2) were consisted of three groups, *i.e.*, Astronyxids (group I), Polyphagids (group II) and Culbertsonids (group III). Cyst of each group has been morphologically

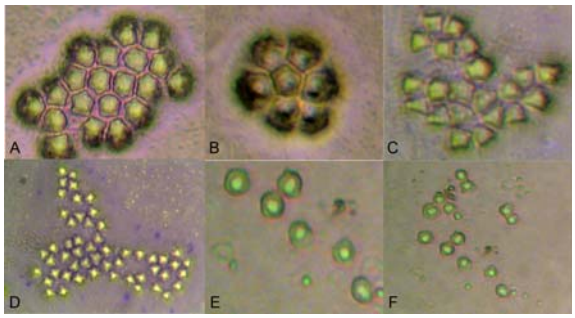

Fig. 1 Cysts of various *Acanthamoeba* spp. growing on non-nutrient agar at day 2
(A), (B): Astronyxids (group I)
(C), (D): Polyphagids (group II)
(E), (F): Culbertsonids (group III)

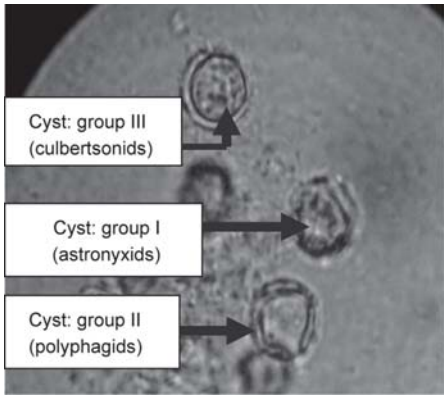

Fig. 2 Three groups of *Acanthamoeba* spp. were developed in contact lens cases at day 5

Table 1. *Acanthamoeba* spp. growing in contact lens cases, the temperature and the characteristics of cysts/trophozoites were recorded for 12 days of observations

Day	Temperature	Results
2	36.8°C	Cysts of various *Acanthamoeba* spp. were grown on non-nutrient agar (day 2) (Fig. 1) (A), (B): Astronyxids (group I) (C), (D): Polyphagids (group II) (E), (F): Culbertsonids (group III)
5	37°C	Three groups of *Acanthamoeba* spp. were developed in contact lens cases at day 5 (Fig. 2)
7	37.1°C	*Acanthamoeba* spp. showing stage of trophozoite emerging from a cyst shell (Fig. 3)
9	37.1°C	The *Acanthamoeba* trophozoites showing contractile vacuole (pale area) and acanthopodia at day 8-9 (Fig. 4)
12	37°C	The *Acanthamoeba* trophozoites freely moved in water and presence of lobopodium and needle like fine projections of pseudopodia called acanthopodia at day 12 (Fig. 5)

described; Astronyxids: species having relatively large cysts with smooth ectocyst and stellate endocyst; Polyphagids: species having wrinkled ectocyst and endocyst, stellate, polygonal, triangular, or oval and Culbertsonids: species typically having thin smooth ectocyst with round endocyst[14]. The average diameter of cysts in group I was relatively large. They were ≤ 18 micron, while those of group II and group III were ≥ 18 micron.

Trophozoites

Acanthamoeba spp showed stage of trophozoite which was emerged from a cyst shell and nuclei at day 7 (Fig. 3). *Acanthamoeba* trophozoites showed a prominent contractile vacuole (pale area) and acanthopodia were well developed at day 8-9 (Fig. 4). The typical morphology of *Acanthamoeba* trophozoites moved freely in water and presence of lobopodium and needle like fine projections of pseudopodia called acanthopodia were presented at day 12 (Fig. 5). The average size of *Acanthamoeba* trophozoites was 25 micron, with in the range of 12-45 micron. Therefore, the life cycle consists of cyst and trophozoite development stages; excystation was shown from image 1 to 10, image no. 10 was shown the 3D view of trophozoite stage (Fig. 6).

Three different images of cyst, a non-stained slide was imaged from optical microscope and AFM (Fig. 7).

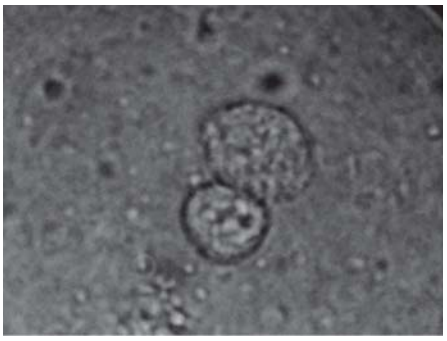

Fig. 3 *Acanthamoeba* spp. trophozoite emerged from a cyst shell and nuclei at day 7

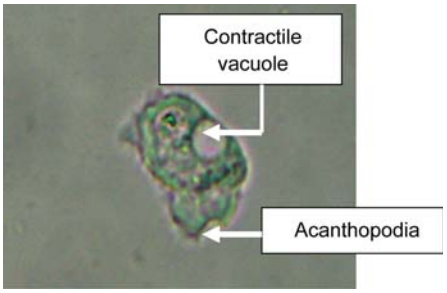

Fig. 4 *Acanthamoeba* trophozoites show contractile vacuole (pale area) and acanthopodia at day 8-9

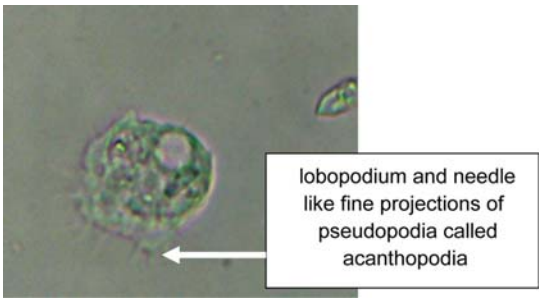

Fig. 5 *Acanthamoeba* trophozoites freely moved in water and presence of lobopodium and needle like fine projections of pseudopodia called acanthopodia at day 12

Discussion

Acanthamoeba spp is ubiquitous free-living protozoa found in a wind range of environmental niches. The authors' pinpoints to only *Acanthamoeba* keratitis (AK) which has been recognized as diseases in humans, and is currently receiving more attention following the association between *Acanthamoeba* and the contact lens wearers. Cysts were identified to 3 different subgenera according to the characteristics previously described[15]. AK has been described primarily from developed countries and several studies suggested that soft contact lens wear would be the greatest risk factor. Singh[15] in 1952, and Singh and Das[16] in 1970, stated that classification of amoebae from locomotion and the appearance of cysts had no phylogenetic value and that these characteristics were not the final diagnostic. They concluded that the mitotic spindle shape was inadequate as a generic character to include *Acanthamoeba* genus. In 1966, *Acanthamoeba spp* became recognized again since Pussard[17] agreed with Singh[15,16] who concluded that spindle shape was an unsatisfactory feature for intergenera differentiation but the distinctive morphology of *Acanthamoeba* cyst was a decisive character at the generic level. In 1967, after the study of several strains of *Hartmannella*, *Acanthamoeba* spp. and other small free-living amoebae, Page[19] concluded that the spindle shape was a doubtful

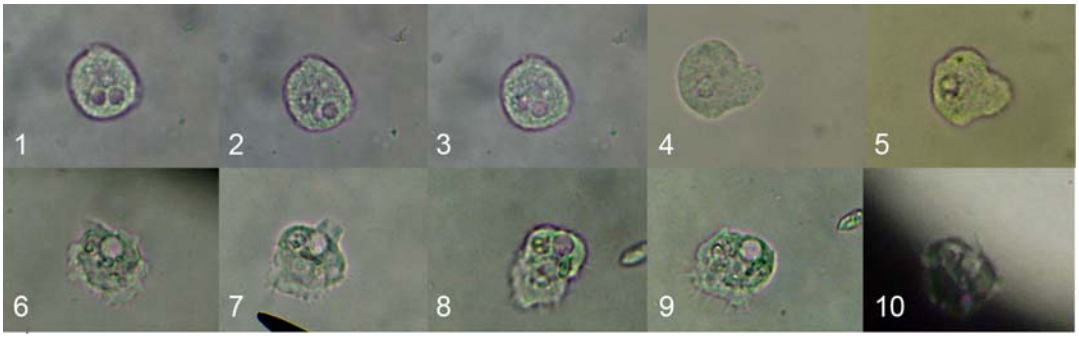

Fig. 6 The life cycle consists of cyst and trophozoite stages, growing of cyst to trophozoite (excystation) (1-10), image No. 10 was shown the 3D view of trophozoite stage

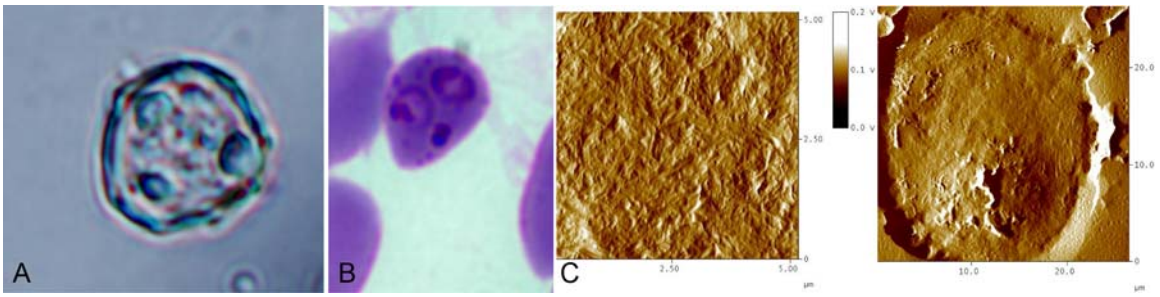

Fig. 7 Cyst for optical microscopy (non-stained) (A), giemsa stained (B) and non-stained for Atomic Force Microscopy (AFM) images; the closer image shown wrinkle scale (C)

criterion for intergeneric differentiation, the presence of acanthopodia and the structure of cyst were be sufficiently distinct. In 1998, Schaumberg et al[19] described eight species of AK; *A. castellanii, A. polyphaga, A. hatchetti, A. culbertsoni, A. rhysodes, A. lugdunensis, A. quina,* and *A. griffini*. When scraped the lesions of corneal abrasion, by direct histopathologic examination and impress cytology culture, they found *Acanthamoeba* trophozoites and cysts as resulted. The most accurate technique for diagnosis of acanthamoebiasis, still requires *in vitro* cultivation which normally takes a few days for trophozoites and one to 2 weeks for encystations. The potential presence of AK is most commonly recognized by the presentation of free-living amoebae, by direct observation of clinical specimens under microscopy[21] and fluorescent antibody or Calcofluor white stain and culture from amoebae inoculation of filtrate on non-nutrient agar[21]. Johnson et al[22] analyzed partial nuclear 18S rRNA sequences from seven isolates of *Acanthamoeba* and obtained results that were concordant with the classification in three morphological groups. Gast et al[23] investigated 18S rRNA gene phylogeny using 18 isolates of *Acanthamoeba* from morphological group II and group III. Luo et al[24] observed the ultra structure of AK in corneal tissue with scanning electron microscope (SEM). Cultured *Acanthamoeba* trophozoites were approximately 15-45 micron in diameter, appeared irregularly round or oval in shape, with rough surface and intrusion of cytoplasm. Culture of the corneal scraping had confirmed *Acanthamoeba* as the etiological agent[25].

The authors have demonstrated that the biofilm phenomenon in contact lens cases which is common in wearers contained AK, despite good compliance with manufacturer's instructions for their lens cleaning system. Biofilm was found more frequently and more densely in contact lens cases; even the instruction and information on the contact lens cases may also provide one explanation for the development of keratitis in wearers of disposable extended wear lenses. It is possible that growth within contact lens cases biofilm is advantageous for the survival of *Acanthamoeba* spp. AK in contact

lens wearers is frequently associated with bacterial biofilm in the contact lens cases. The results of *Acanthamoeba* spp. cultivation in contact lens cases and on non-nutrient agar shown that the *Acanthamoeba* spp. could be grown at 36.8-37.2°C. Bowers[26] indicated that *Acanthamoeba* cysts are typically 10-25 micron in diameter. The cysts have two walls: a wrinkled fibrous outer wall (exocyst) and an inner wall (endocyst) that may be hexagonal, spherical, star-shaped or polygonal. Cysts contain only one nucleus with a large karyosome. *Acathamoeba* trophozoites were measured approximately 15-45 micron and they present a Golgi complex, smooth and rough endoplasmic reticula, free ribosomes, digestive vacuoles, mitochondria, and microtubules, contain a large nucleus with a large, centrally-located karyosome but no peripheral chromatin. A trilaminar plasma membrane was found to surround the cytoplasmic contents of the trophozoite. In addition, distinguishing features of the trophozoite were the presence of spiny surface projections called acanthopodia and a nucleus with a large central nucleolus. A double-walled wrinkled cyst is composed of an ectocyst and an endocyst ranges in size from 13 to 20 micron. Three different images of cyst, in this study, a non-stained slide was imaged. The fixed-trophozoite could not be scanned by AFM because the cantilever to find out was not suitable for the cell surface. The cell height of membrane surface which may be not a flat surface was too close to the cantilever tip of AFM. Height and spring constant of AFM cantilever are critical for the imaging of living cell. However, the contact lens cases care system must be health safety tested against bacteria in the biofilm before being licensed. The observations by biofilm scraping from contact lens cases must be identified and differentiated accurately for these three different *Acanthamoeba* groups. As well as the confirmation strategy using non-nutrient cultivation method in agar would be the proper of cysts and trophozoites morphological characteristics study.

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รูปลักษณะของเชื้ออะคันตามีบาที่เป็นสาเหตุของโรคกระจกตาอักเสบจากตลับใส่คอนแทคเลนส์ติดเชื้อ

อุษา เล็กอุทัย, รังสิมา พัสระ, โกศล รุ่งเรืองชัย

วัตถุประสงค์*: เพื่อศึกษารูปลักษณะของเชื้ออะคันตามีบาที่เป็นสาเหตุของโรคกระจกตาอักเสบจากตลับใส่คอนแทคเลนส์ที่ติดเชื้ออะคันตามีบา โดยวิธีเพาะเชื้อ*

วัสดุและวิธีการ*: เก็บตัวอย่างด้วยการขูดฟิล์มบาง ๆ ข้างตลับใส่คอนแทคเลนส์ จำนวน 150 ตลับของนักศึกษาในสถาบันการศึกษาแห่งหนึ่งที่จังหวัดนครปฐมระหว่างเดือนมีนาคมถึงเดือนกันยายน พ.ศ. 2550 นำมาเพาะเชื้อด้วยวิธีมาตรฐานเพื่อสังเกตการพัฒนาการออกจากเกราะของซิสต์ โดยซิสต์ของอะคันตามีบาที่มีน้ำยาแช่และมี Escherechia coli นำไปบ่มที่อุณหภูมิประมาณ 37 องศาเซลเซียส สังเกตการพัฒนาของเชื้อระยะซิสต์เป็นโทรโฟซอยท์และจดบันทึกการศึกษารูปร่างลักษณะของซิสต์และการพัฒนาการของซิสต์เป็นโทรโฟซอยส์ด้วยกล้องจุลทรรศน์นาน 12 วันรวมทั้งการเปรียบเทียบรูปร่างซิสต์แบบย้อมและไม่ย้อมสี

ผลการศึกษา*: การสำรวจพบอัตราความชุกของการติดเชื้อที่ตลับคอนแทคเลนส์ ร้อยละ 6.7 (10/150) และพบลักษณะอะมีบาที่มีรูปร่างต่างกัน 3 กลุ่มที่อุณหภูมิ ตั้งแต่ 36.8-37.2 องศาเซลเซียส ประกอบด้วยกลุ่ม 1: Astronyxids, กลุ่ม 2: Polyphagids และกลุ่ม3: Culbertsonids ขนาดซิสต์เฉลี่ยในกลุ่ม1 มีขนาดใหญ่ที่สุดคือประมาณ 18 ไมครอน หรือใหญ่กว่าเล็กน้อย ส่วนกลุ่ม 2 และกลุ่ม 3 มีขนาดซิสต์เฉลี่ยน้อยกว่า 18 ไมครอน สำหรับลักษณะจำเพาะของโทรโฟซอยต์ที่เคลื่อนไหวอิสระในน้ำขณะทดสอบ 12 วัน มีลักษณะการยื่นขาเทียมและแขนงแหลมคล้ายเข็ม (acanthopodia) โดยมีขนาดประมาณ 25 ไมครอน (12-45 ไมครอน) แสดงภาพลักษณะภายนอกของซิสต์ที่ย้อมและไม่ย้อมด้วยกล้องจุลทรรศน์*

สรุป*: เชื้ออะคันตะมีบาเป็นสาเหตุของโรคกระจกตาอักเสบ (Acanthamoeba keratitis) โดยเฉพาะผู้ใส่คอนแทคเลนส์ที่ไม่สะอาด สามารถสังเกตการพัฒนาการออกจากเกราะของซิสต์ (excystation) ด้วยการเพาะเชื้อในวิธีมาตรฐานในเอกา (agar) ที่ไม่ต้องมีอาหารลี้ยงเชื้อ การจำแนกกลุ่มอะคันตามีบาทั้ง 3 กลุ่มจากการขูดฟิล์มบางในตลับใส่คอนแทคเลนส์ ตามด้วยการตรวจด้วยการจำแนกรูปร่างลักษณะของซิสต์และโทรโฟซอยต์ของเชื้อ ดังนั้นการให้สุขศึกษาและการดูแลตลับใส่คอนแทคเลนส์ที่ถูกสุขอนามัยที่ดี และการใช้คอนแทคเลนส์ชนิดใช้ครั้งเดียวอาจแก้ปัญหาการติดเชื้อจากตลับใส่คอนแทคเลยส์ได้*