DETECTION OF ACANTHAMOEBA KERATITIS FROM CONTACT LENS STORAGE CASES USING LOOP-MEDIATED ISOTHERMAL AMPLIFICATION METHOD

RANGSIMA PASSARA

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Thesis

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....

Miss Rangsima Passara

Candidate

....

Assoc.Prof. Dr. Usa Lek-Uthai,

Dr.P.H.(Public Health)

Major-Advisor

....

Assoc.Prof. Nitaya Thammapalerd,

M.Sc.(Biochemistry)

Co-Advisor

....

Prof. Banchong Mahaisavariya, M.D.

Dean

Faculty of Graduate studies

....

Assoc.Prof. Kanokrat Siripanichgon,

B.Sc.(PT), M.D., M.P.H.

Chair

Master of Science (Public Health)

Major in Infectious Diseases and Epidemiology

Faculty of Public Health

Thesis

Entitled

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For the degree of Master of Science (Public Health)

Major in Infectious Diseases and Epidemiology

on

December 26, 2007

Rangsima Passara

....

Miss Rangsima Passara

Candidate

....

Dr. Kawin Leelawat,

M.D., Ph.D. (Anatomy)

Chair

Nitaya Thammapalerd

....

Assoc.Prof. Nitaya Thammapalerd,

M.Sc.(Biochemistry)

Member

Usa Lek-Uthai

....

Assoc.Prof. Dr. Usa Lek-Uthai,

Dr.P.H.(Public Health)

Member

B. Mahaisavariya

....

Prof. Banchong Mahaisavariya,M.D

Dean

Faculty of Graduate studies

Mahidol University

Phitaya Ch, M.D.

....

Assoc.Prof. Phitaya Charupoonpol,

M.D., Dip.Thai Board (Epidemiology)

Dean

Faculty of Public Health

Mahidol University

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Rangsima Passara

DETECTION OF ACANTHAMOEBA KERATITIS FROM CONTACT LENS STORAGE CASES USING LOOP-MEDIATED ISOTHERMAL AMPLIFICATION METHOD

RANGSIMA PASSARA 4837529 PHPH/M

M.Sc.(PUBLIC HEALTH)MAJOR IN INFECTIOUS DISEASES AND EPIDEMIOLOGY

THESIS ADVISORS: USA LEK-UTHAI, Dr.P.H., NITAYA THAMMAPALERD, M.Sc.(BIOCHEMISTRY)

ABSTRACT

The genus *Acanthamoeba* is an opportunistic organism associated with wearing contact lenses. The main objectives of this study were to detect *Acanthamoeba* using the Loop-mediated isothermal amplification method (LAMP) from contact lens storage cases and to compare the results with the ‘gold standard’ culture methods. This study was conducted from March until October 2007, at Silapakorn University, Nakhornpathom Province. Laboratories were used at the Faculty of Public Health, Department of Parasitology, Mahidol University, Bangkok. The prevalence of *Acanthamoeba* spp. in contact lens wearers was 6.7 % (10/150) using culture technique, and the proper *Acanthamoeba* spp. trophozoite growth was found between 36.8-37.2°C. The *Acanthamoeba* spp. detected by cultivation and LAMP methods were shown to be positive. The LAMP optimized temperature was fitted to 65°C. The appropriate visual turbidity precipitated identification was performed within 10 minutes after the reaction end. The size of DNA products from both LAMP and Real-time PCR methods were 175 bp. Since the outer primer pair of LAMP primers can also be used for Real-time PCR method, the lower limit of detection of LAMP and Real-time PCR were compared by making serial dilutions of genomic DNA. Comparison of lower limits of detection in LAMP and Real-time PCR Assay indicated that the former was able to detect *Acanthamoeba* spp. DNA dilutions up to 10 pg while the latter up to 100 pg. However, when comparing the cost of each technique, the culture technique was found to be the cheapest at 4.20 Baht, but LAMP took less time, approximately 2 hrs. The sensitivity and specificity of the LAMP and culture techniques were equal at 100%. LAMP will still be useful for the emergency initial screening of suspected infections caused by *Acanthamoeba* spp., the important causative agents of *Acanthamoeba* keratitis in contact lens wearers. This LAMP method can be an early and rapid detection method for prompt treatment.

KEY WORDS: ACANTHAMOEBA KERATITIS/ CONTACT LENS STORAGE CASES/ LOOP-MEDIATED ISOTHERMAL AMPLIFICATION METHOD

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(DETECTION OF ACANTHAMOEBA KERATITIS FROM CONTACT LENS STORAGE CASES USING LOOP-MEDIATED ISOTHERMAL AMPLIFICATION METHOD)

รังสิมา พัสระ 4837529 PHPH/M

วท.ม. (สาธารณสุขศาสตร์) สาขาวิชาเอกโรคติดเชื้อและวิทยาการระบาด

คณะกรรมการควบคุมวิทยานิพนธ์: อุษา เล็กอุทัย, Dr.P.H., นิตยา ธรรมพาเลิศ M.Sc.(BIOCHEMISTRY)

บทคัดย่อ

เชื้ออะแคนทามีบา เป็นอะมีบาสิ่งแวดล้อมและเป็นเชื้อฉวยโอกาสทำให้เกิดโรคหรือไม่เกิดโรคในคน โดยมักพบการติดเชื้อที่กระจกตาในผู้ที่ใช้คอนแทคเลนส์ การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อตรวจหาเชื้ออะแคนทามีบา โดยเปรียบเทียบระหว่างวิธีการเพาะเลี้ยงเชื้อและวิธีการเพิ่มจำนวน ดี เอ็น เอ ด้วยวิธี Loop-mediated isothermal amplification method (LAMP) จากการเก็บตัวอย่างตลับคอนแทคเลนส์จากนักศึกษามหาวิทยาลัยศิลปากร วิทยาเขตพระราชวังสนามจันทร์ที่ใช้คอนแทคเลนส์ จำนวน 150 ชิ้น ตั้งแต่เดือนมีนาคมถึงตุลาคม 2550 ผลการศึกษาโดยวิธีเพาะเชื้อและวิธี LAMP พบเชื้ออะแคนทามีบาในตัวอย่างตลับคอนแทคเลนส์ 10 ตัวอย่างคิดเป็นร้อยละ 6.7 (10/150) ซึ่งการเพาะเชื้อพบว่า เชื้ออะแคนทามีบาเจริญเติบโตที่อุณหภูมิระหว่าง 36.8-37.2°C และอุณหภูมิ 65°C เป็นอุณหภูมิที่เหมาะสมที่สุดในปฏิกิริยาของวิธี LAMP และสามารถอ่านผลได้ชัดเจนภายใน 10 นาทีหลังสิ้นสุดปฏิกิริยา วิธีนี้มีความไวและความจำเพาะสูง อ่านผลได้ด้วยตาเปล่า และสามารถใช้ primer คู่นอกของวิธี LAMP เพื่อเปรียบเทียบปริมาณ DNA กับวิธี Real-time PCR ซึ่งพบว่า วิธี LAMP สามารถตรวจพบปริมาณ DNA ได้ต่ำสุดที่10 pg ส่วนวิธี Real-time PCR สามารถตรวจพบปริมาณDNA ได้ต่ำสุดที่ 100 pg เมื่อเปรียบเทียบค่าใช้จ่ายในแต่ละวิธีพบว่า วิธีการเพาะเลี้ยงเชื้อเสียค่าใช้จ่ายน้อยที่สุดเพียง (4.20 บาท) วิธีเพาะเชื้อและวิธี LAMP มีความไวและความจำเพาะเท่ากับร้อยละ 100 แต่เมื่อเปรียบเทียบเวลาที่ใช้พบว่า LAMPใช้เวลาน้อยที่สุดคือ 2 ชั่วโมง ดังนั้นจึงสามารถนำวิธี LAMP มาใช้ในการตรวจหาเชื้ออะแคนทามีบาในรายที่มีอาการรุนแรงและมีความจำเป็นต้องได้รับการตรวจรักษาอย่างเร่งด่วน ซึ่งสามารถทำให้การตรวจวินิจฉัยแยกโรคเป็นไปอย่างถูกต้องและรวดเร็ว ทำให้ผู้ป่วยได้รับการรักษาอย่างถูกวิธี และช่วยลดการวินิจฉัยที่ผิดพลาดได้ ผลการศึกษาครั้งนี้จะเป็นประโยชน์ต่อการเฝ้าระวังการติดเชื้อ และการรักษาเชื้ออะแคนทามีบาในผู้ที่ใช้คอนแทคเลนส์ได้

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ABBREVIATIONS

A	=	*Acanthamoeba* spp.
bp	=	base pairs
DNA	=	Deoxyribonucleic acid
dNTP	=	Deoxynucleoside triphophate
et al.	=	Et alli-and other
i.e.	=	Id est- that is
Kb	=	Kilobase
LAMP	=	Loop-mediated isothermal amplification
M	=	Molar
mg	=	Milligram
ml	=	Milliliter
μg	=	Microgram
min	=	Minute
μ	=	Micron
μm	=	Micrometer
μl	=	Microliter
ng	=	Nanogram
pmol	=	picomole (10^{-12}g)
PAGE	=	Polyacrylamide gel electrophoresis
pH	=	Negative logarithm of hydrogen ion activity
RFLP	=	Restriction-Fragment-Length Polymophisms
rpm	=	Revolutions per minute
spp.	=	Species
Tm	=	Melting Temperature
C_T	=	Threshold cycle
V	=	Volt
× g	=	Acceleration gravity

ABBREVIATIONS (CONT)

%	=	Percent
°C	=	Degree Celsius
w/v	=	Weight per volume
λ	=	Lambda

CHAPTER I
INTRODUCTION

Background and rationale

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 disease of the central nervous system known as granulomatous amoebic encephalitis (GAE), a sight-threatening eye disease known as *Acanthamoeba* keratitis and secondary infection of skin lesions associated with immunocompromised individuals, such as AIDS patients (1).

Acanthamoeba keratitis affects healthy persons, both contact lens users and non-contact lens users. The disease was first described in the United States in 1973 (2) and then several hundreds of cases were reported worldwide. In the United Kingdom, the incidence of *Acanthamoeba* keratitis has risen since 1984, which can arise from accidental trauma to the cornea. Contact lens wearers are most at risk from infection and account for 62-71% of *Acanthamoeba* keratitis cases (3). Lack of good hygiene to handle the lens, notably the use of non-sterile saline rinsing solutions, and defaulting the practice of the recommended cleaning and disinfection steps are recognized as risk factor (3). Biofilms are known to play an important role in the pathogenesis of *Acanthamoeba* keratitis. Biofilms are microbially derived sessile communities, which can be formed in aqueous environments as well as on any materials and medical devices including intravenous catheters, contact lenses, scleral buckles, suture material and intraocular lenses (4). With contact lenses, biofilms are formed through contamination of the storage case. Once established, biofilms provide attractive niches for *Acanthamoeba*, by fulfilling their nutritional requirements as well as providing resistance to disinfectants. For example, Beattie *et al*.(5) have shown that *Acanthamoeba* exhibit significantly higher binding to use and *Pseudomonas* biofilm coated hydrogel lenses compared with unworn contact lenses.

In addition, the abundant nutrient provided by the biofilm encourages transformation of *Acanthamoeba* into the vegetative, infective trophozoite form, and it is important to remember that binding of *Acanthamoeba* keratitis to human corneal epithelial cells most likely occurs during the trophozoite stage as cysts exhibit no and/or minimal binding (6). Overall, these findings suggest that biofilms play an important role in *Acanthamoeba* keratitis in wearers of contact lenses and preventing their formation is an important preventive strategy.

In Thailand, Roongruangchai and Supadirekkul (7) reported 2.4% (2/87) prevalence of *Acanthamoeba* spp. in contact lens cases. Kosrirukvongs *et al*. (8) reported 6 cases of *Acanthamoeba* keratitis at Siriraj Hospital from January to October 1999 and they were treated with Chlorhexidine. Jongwutiwes and colleagues (9) identified the first, two keratitis patients at Chulalongkorn Memorial Hospital on June 15, 1988 and March 12, 1990. Contaminated contact lens care systems usually are the first step in *Acanthamoeba* keratitis pathogenesis. The most prevalent risk factors are contact lens wear, poor hygiene, and a compromised corneal barrier. Users of extended wear lenses are at special risk. Unfortunately, due to the very often misdiagnosis and the complicated treatment, *Acanthamoeba* keratitis frequently gets a serious progression that may cause serious visual loss and perforating keratoplasty. Therefore, fast and reliable diagnosis is of crucial importance. *Acanthamoeba* keratitis is easily confused with atypical herpes simplex keratitis or fungal keratitis. Clinical diagnosis should be based on the presence of keratitis with severe pain and photophobia, ring like stromal infiltrates, radial keratoneuritis, and sometimes pseudodendriform epithelial lesions. Cysts or trophozoites, found in corneal scrapings, on contact lenses, and inside of lens storage cases, are confirmatory. Agar culture is the mainstay for laboratory detection of *Acanthamoeba* (10). Despite culture techniques that require familiarity with the morphology of cysts and trophozoites of *Acanthamoeba*, and it may take 1 to 10 days. Therefore, in the face of negative culture, a delay of several days in diagnosis is involved, thus leading to a delay in instituting specific therapy.

As the Loop-mediated isothermal amplification method shown high sensitivity (95%) and high specificity (99%) for *Plasmodium falciparum* detection from heat-treated blood and it did not require thermal cyclers, expensive reagents and the

interpretation of results does not require highly experienced staff because the assay positivity can be verified by visual inspection (11). Comparatively, the Loop-mediated isothermal amplification method can be easily added to the armamentarium of diagnostic methods in a microbiology laboratory. Additionally, the short time taken by the Loop-mediated isothermal amplification method is a distinct advantage over the culture method. Hence, we hope that the Loop-mediated isothermal amplification method would be very helpful and desirable for rapid diagnosis of *Acanthamoeba* keratitis and would be a confirm method in the detection of clinically suspected cases with or without culture.

In this study we will use the Loop-mediated isothermal amplification method for detection and identification of *Acanthamoeba* keratitis from contact lens storage cases and compare the result with culture methods from the same samples and compare the sensitivity and specificity of these results. Since the outer primer pair of LAMP primers can also be used for Real-time PCR, so in this study we compared the lower limit of detection of LAMP and Real-time PCR by serially dilution.

General objective

To detect *Acanthamoeba* spp. using Loop-mediated isothermal amplification method.

Specific objectives

1. To observe the proper temperature for *Acanthamoeba* spp. growth in contact lens storage cases.
2. To know the prevalence of *Acanthamoeba* spp. From contact lens storage cases in students at Silapakorn University.
3. To compare the sensitivity and specificity of the Loop-mediated isothermal amplification method and culture technique for detection of *Acanthamoeba* spp. from contact lens storage cases.
4. To compare the sensitivity of the two methods: the Loop-mediated isothermal amplification method and Real-Time PCR Assays to the lower limit of detection of *Acanthamoeba* spp.

Definitions

***Acanthamoeba* spp.:** A ubiquitous free-living protozoa, occurring in two forms, *i.e*., trophozoites and non motile cysts. Occurrence in the cyst form accounts for its resistance to unfavorable environments including chemotherapy.

***Acanthamoeba* keratitis:** *Acanthamoeba* keratitis is a painful progressive sight-threatening corneal disease. Several species of *Acanthamoeba*, including *A. castellanii, A. polyphaga, A. hatchetti, A. culbertsoni, A. rhysodes, A. griffini, A. quina, and A. lugdunensis*, have been reported to cause AK. In this study *Acanthamoeba* keratitis was meant *Acanthamoeba* spp. that causes keratitis.

Cysts of *Acanthamoeba*: A non-feeding and non-motile form of *Acanthamoeba* spp. having resistant double wall, an outer ectocyst and an inner endocyst.

Trophozoites of *Acanthamoeba*: A sluggishly motile feeding form of *Acanthamoeba* at the average diameter of 25 μm with little evidence of progressive motility. Pseudopodia consist of lobopodia together with needle-like acanthopodia. *Acanthamoeba* trophozoites contain one nucleus with a central karyosome.

Loop-mediated isothermal amplification (LAMP): A novel method that amplifies DNA with high specificity, efficiency and rapidity under isothermal conditions. This method employs a DNA polymerase and a set of four specially designed primers that recognize a total of six distinct sequences on the target DNA.

Real-Time PCR: Real-Time PCR is a variation of PCR that combines the amplification ability of PCR with a fluorescent detection system for analysing results as a product as made. In this study, SYBR Green I was used. The fluorescent label significantly enhances our ability to detect the product of PCR and thus increases the sensitivity of the reaction. The equipment used for RT-PCR was Chromo 4 TM System (Bio-Rad, U.S.A).

QuantiTect SYBR Green PCR Master Mix: QuantiTect SYBR Green PCR Master Mix contains an optimized concentration of the fluorescent dye SYBR Green I. SYBR Green I binds all double-stranded DNA molecules, emitting a fluorescent probes. The excitation and emission maxima of SYBR Green I are at 494 nm and 521 nm, respectively, Which are compatible with use on any real-time cycler.

Melting curve analysis: Every piece of dsDNA has a melting point (T_m) at which temperature 50% of the DNA is single stranded. The temperature depends on the length of the DNA, sequence order, G:C content and Watson-Crick pairing. When DNA-binding dyes are used, as the fragment is heated, a sudden decrease in fluorescence is detected when T_m *is* reached (due to dissociation of DNA strands and release of the dye). This point is determined from the inflection point of the melting curve or the melting peak of the derivative plot.

Contact lens storage cases: The storage cases of contact lens. With contact lenses, biofilms are formed through contamination of the storage case, biofilms provide attractive niches for *Acanthamoeba*, by fulfilling their nutritional requirements as well as providing resistance to disinfectants.

Cultivation Method: Isolation of *Acanthamoeba* using non-nutrient agar plates seeded with Gram-negative bacteria. In this study, we used 1.5% non-nutrient agar plates seeded with *Escherichia coli* as food source for *Acanthamoeba.* The non-nutrient agar contains minimal nutrients and thus inhibits the growth of unwanted organisms.

LAMP Primers: *Acanthamoeba* spp. specific LAMP primers set consisting of primers F3, B3, FIP and BIP was designed using the Primer Explorer program to amplify the 18S rRNA from GenBank (accession no.AY694145.1 *Acanthamoeba* spp. 18S ribosomal RNA gene)

Biofilm Scraping: Biofilms are microbially derived sessile communities, which can be formed in aqueous environments as well as on any materials and medical devices including intravenous catheters, contact lenses, scleral buckles, suture material and intraocular lenses. In this study, the contact lens storage cases were opened under aseptic conditions. The amoeba saline was dropped into the contact lens storage cases and scraping with sterile cotton bud over the internal surface of them.

CHAPTER II
LITERATURE REVIEW

Free-living amoebae are opportunist pathogenic to humans such as *Naegleria* spp., *Acanthamoeba* spp. and *Balamuthia mandrillaris*, which are found in a wide range of environmental niches. They are resistant to disinfectants, temperature variation and desiccation. These organisms are recognized diseases in humans including Primary Amoebic Meningoencephalitis (PAM) (12), granulomatous amoebic encephalitis (GAE) (13) and *Acanthamoeba* keratitis (AK) (14) respectively. The latter is currently receiving more attention following the association between *Acanthamoeba* and the wearing of contact lens. Laboratory diagnosis is beyond the scopes of review on the various aspects including the ecology, classification, pathogenicity, and laboratory detection of *Acanthamoeba* spp.

Classification of *Acanthamoeba*

The first *Acanthamoeba* was isolated in 1912 by Alexeieff (15) and genus was established in 1931 and there are considerable confusions in the literature and taxonomic status since that time. *Castellanii* discovered as an amoeba in the culture of the fungus *Cryptococcus pararosus* (16). Douglas (16) places this amoeba in the genus *Hartmannella* and named it as *Hartmannella castellanii*. Volkonsky (17) in 1931, considered the genus *Hartmannella* to be an artificial assemblage of unrelated amoebae and subdivided it into three generas. All amoebae were characterized by round, smooth-walled cysts and those that contain a cylindrical or truncated spindle are placed in the genus *Hartmannella*. The amoebae characterized by nuclear division in the cyst were in the *Glaesaria*.

Volkonsky (17) created the genus *Acanthamoeba* for amoebae characterized by the appearance of pointed spindles at mitosis and that have double-walled cysts with ostioles and an irregular outer layer. Singh (18) in 1952, and Singh and Das (19) 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 shape of the mitotic spindle was inadequate as a generic character to include the genus *Acanthamoeba.*

In 1966, the genus *Acanthamoeba* became recognized again since Pussard (20) agreed with Singh's conclusion that spindle shape was an unsatisfactory feature for intergenera differentiation but the distinctive morphology of the cyst was a decisive character at the generic level. In 1967, after studying several strains of *Hartmannella*, *Acanthamoeba* and other small free-living amoebae, Page (21) concluded that the shape of the spindle was a doubtful criterias for intergeneric differentiation but considered the presence of acanthopodia and the structure of the cyst to be sufficiently distinctive. The generic designations of *Hartmannella* and *Acanthamoeba* were justified because the genus *Hartmannella* has nothing in common with the *Acanthamoeba* except for a general mitotic pattern which is a property shared with many other amoebae. In 1975, Visvesvara and Balamuth (22) clearly identified the definable and demonstrable differences between *Acanthamoeba* and *Hartmannella*, including morphological variation from nutritional requirements and serologic responses. In 1979, Singh and Hanumaiah (23) accepted the genus *Acanthamoeba*, thereby concurring with Page *et al* (21), but they placed the genus in the family *Hartmannellidae.* In 1975, Sawyer and Griffin (24) created the family *Acanthamoebidae*. *Hartmannella* was placed in the family *Hartmannellidae,* order Euamoebida. The position of *Acanthamoeba* in the taxonomic scheme of the Society of Protozoologists is shown in Table 1.

Teble 1 Taxonomic classification of the *Acanthamoeba* (25)

Kingdom	**Protista**	**Taxonomic characteristics**
Subkingdom	Protozoa	Unicellular
Phylum	Sarcomastigophora	Amoeboid form
Subphylum	Sarcodina	
Superclass	Rhizopodea	Locomotion by lobopodia
Order	Amoebida	Uninucleate, mitochondria present, no flagellate stage
Suborder	Acanthopodina	More or less finely tipped, sometimes filiform, often furcate hyaline subpseudopodia produced from a broad hyaline lobe not regularly
Family	Acanthamoebidae	
Genus	*Acanthamoeba*	
Species	Isolated from human *A. astronyxis* (CNS) *A. castellanii* (CNS, eye) *A. culbertsoni* (CNS, eye) *A. hatchetti* (Eye) *A. palestinensis* (eye) *A. polyphaga* (CNS, eye)	Not Isolated from human *A. comandini* *A. griffini* *A. lenticulata* *A. royreba* *A. tubiashi* *A. species*

Life Cycle and Characteristic of *Acanthamoeba*

The small free-living amoebae are classified under *Acanthamoebidae* (*Acanthamoeba*) and *Vahlkampfidae* (*Naegleria*). Identifications are based primarily on morphology, nuclear divisions, temperature tolerance, isoenzyme pattern, and pathogenicity (26). As more precise immuno-chemical and molecular biological tools, such as monoclonal antibodies and restriction endonuclease digestion of cellular DNA, have become available, periodic revisions of the classification of this group of protozoa will be necessary (27).

Acanthamoeba differs from *Naegleria* by several means. *Acanthamoeba* has only two developmental stages, *i.e*, trophozoites and cysts in their life cycle while *Naegleria* has three developmental stages, *i.e*, trophozoites, flagellates (bi-or more) and cysts. Each trophozoite is also characterized by a single nucleus and a large karyosome. The motility of *Acanthamoeba* is sluggish; instead of forming broad lobopodia, it forms spiny or filose pseudopods called acanthopodia. The amoeba feeds on bacteria such as *Escherichia coli, Enterobacter* spp. and other enteric gram negative bacilli. Some species may engulf *Legionella pneumophilla* (28), blue green algae, or yeasts. In an actively growing culture, a large contractile vacuole is clearly visible as it empties and reappears within a few minutes. Subgenera divided into three groups by size and morphology of cysts by Pussard and Pons (20) and Visvesvara (22) and a list group of *Acanthamoeba* spp. (Table 2).

Group I Astronyxids : The average diameter of the cysts is larger than 18 μm. The ectocyst and the endocyst are widely separated. The outer cyst wall is smooth or gently wrinkled, the endocyst is more or less stellate, the endocyst meets the ectocyst at the ends arms or rays, and the operculum is normally at the level of the ectocyst.

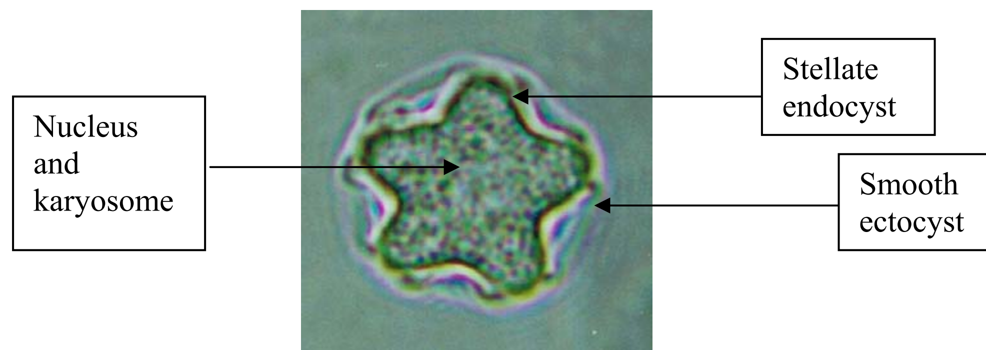


Figure 1 Cysts of group I (Astronyxids) (17)

Group II Polyphagids: is the largest group and includes 10 species. The mean diameter of the cysts is usually less than 18 μm. The group includes the most widespread and commonly isolated Acanthamoebae. Many of the Acanthamoebae isolated from corneal biopsy specimens, smears, and contact lens paraphernalia belong to this group. The ectocyst and the endocyst are either close together or widely separated. The ectocyst may be thick or thin and is usually wrinkled or mamillated. The endocyst may be stellate, polygonal, triangular, or sometimes round or oval and usually does not have well-developed arms or rays. The operculum, at the junction of the endocyst and ectocyst, is in a depression formed by in folding of the ectocyst.

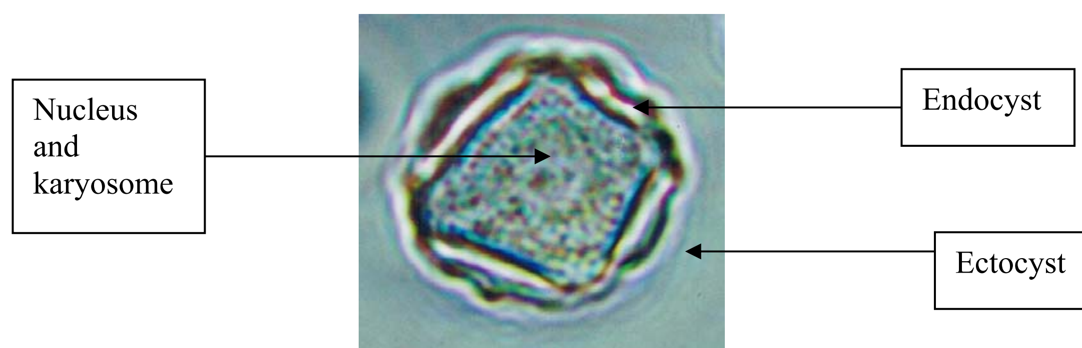


Figure 2 Cysts of group II (Polyphagids) (17)

Group III Culbertsonids : The mean diameter of the cyst is usually less than 18 µm. The ectocyst in this group is thin and is either gently rippled or unrippled. The endocyst is usually round but may have three to five gentle corners. It may not be possible to identify the species within this group by morphology alone.

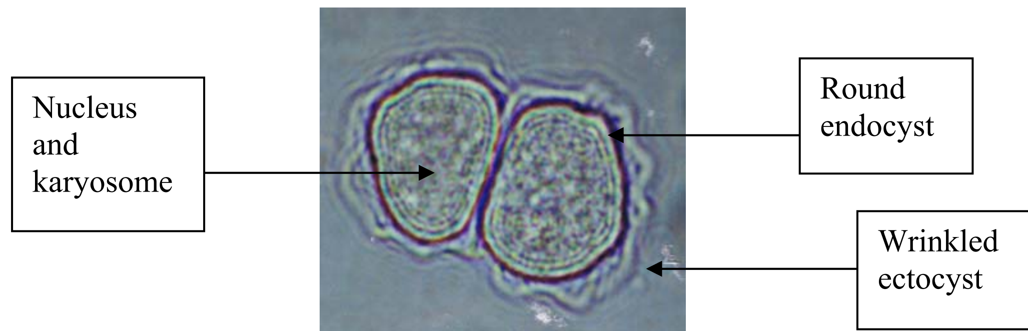


Figure 3 Cysts of group III (Culbertsonids) (17)

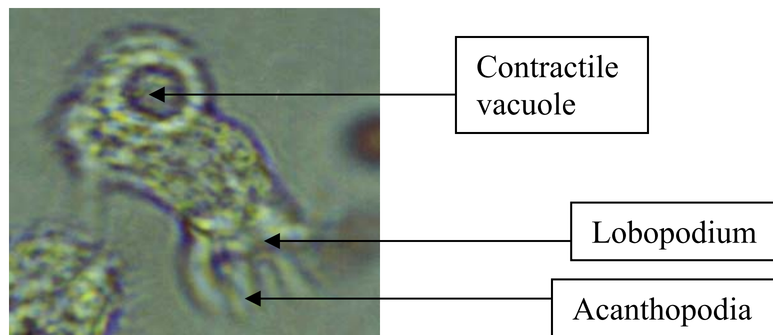


Figure 4 Trophozoite of *Acanthamoeba* spp. (17)

Table 2 Groups of *Acanthamoeba* species (25)

Group I **Astronyxids**	**Group II** **Polyphagids**	**Group III** **Culbertsonids**
A. astronyxis *A. comandoni* *A. echinulata* *A. tubiashi*	*A. castellanii* *A. divionensis* *A. griffini* *A. hatchetti* *A. lugdunensis* *A. mauritaniensis* *A. polyphaga* *A. quina* *A. rhysodes* *A. triangularis*	*A. culbertsoni* *A. lenticulata* *A. royreba* *A. palestinensis*

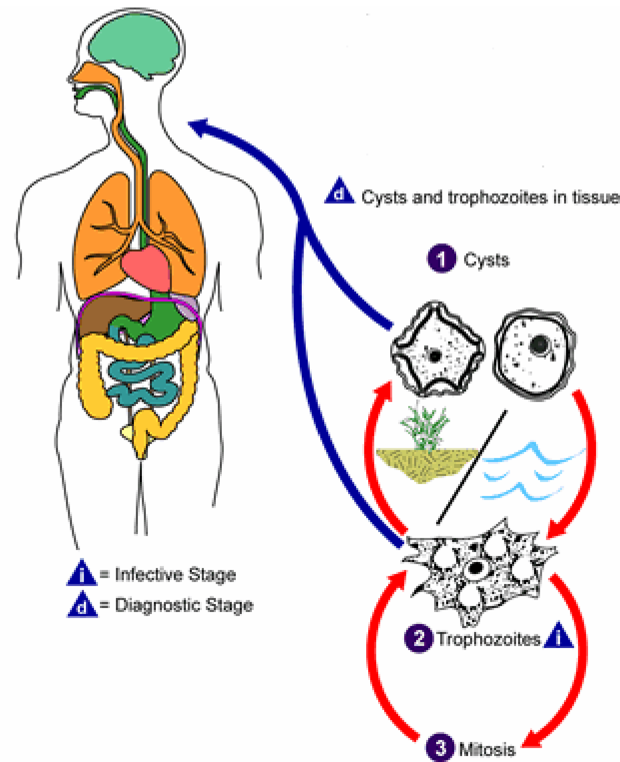


Figure 5 Life cycle of *Acanthamoeba* spp.

Source: http: // www.dpd.cdc.gov/dpdx/HTML/Image_Libraly.htm [Accessed Dec. 7, 2006]

Clinical manifestations

The route of invasion and penetration into the CNS in cases of GAE appears to be hematogenous, probably originating at a primary focus in the skin or respiratory tracts (lung). The neuropathologic changes consist of chronic granulomatous encephalitis with multinucleated giant cells mainly on the posterior fossa structures, basal ganglia, base of the cerebral hemispheres, and cerebellum. Trophozoites and cysts may be found within the CNS lesions. Giant cells may not be present in immunosuppressed individuals (29).

In the last decade, *Acanthamoeba* keratitis is an entirely unknown entity. It has developed into a distinct, although still uncommon, ophthalmic problem. It is now established that *Acanthamoeba* can directly infect the cornea (generally after trauma associated with contaminated water or contact lenses) causing extremely serious

keratitis. This infection is highly resistant to therapy often resulting in marked visual impairment or even blindness. More recent cases related to contact lens wearers, would certainly appear to be the combined result of microscopic epithelial trauma and contamination of contact lens washing solution, such as homemade saline solution.

In 1998, Schaumberg *et al.*(30) described eight species of *Acanthamoeba* that have been identified in amoebic keratitis (*A.castellanii, A. polyphaga, A. hatchetti, A. culbertsoni, A. rhysodes, A. lugdunensis, A. quina, A. griffini*). When trauma is severe enough to cause corneal abrasion, a more rapid process usually develops to corneal ulceration, increasing corneal infiltration and ring clouding, iritis, and often scleritis, severe pain, hypo yon, and marked loss of vision. When the condition occurs in contact lens wearers, the symptoms usually begin more complex but progresses just as inexorable. Where no obvious trauma has occurred, the early corneal findings may be nonspecific or suggest as treated as *Herpes simplex* infection. In some patients the condition waxes and wanes appeared to clear completely. It may reappear suddenly as a rapid progressing corneal abscess characterized by a unique ring-shaped of define diagnostic importance (31). When scraped, the lesions were found, by direct histopathologic examination and impression cytology culture, to contain *Acanthamoeba* trophozoites and cysts (32).

Acanthamoeba keratitis is generally regarded as a chronic condition manifested by ring and per neural infiltrate, typically found in contact lens wearers. Although known to resemble *Herpes* keratitis with dendriform-punctate stroma (immunogenic) and neurotrophic presentations, *Acanthamoeba* is rarely detected in the early phases of its diseases. After the recognition of home-made normal saline contact lens solutions as a principle source of *Acanthamoeba* corneal infection, the incidence has stabilized to an infrequent external cause of keratitis in most.

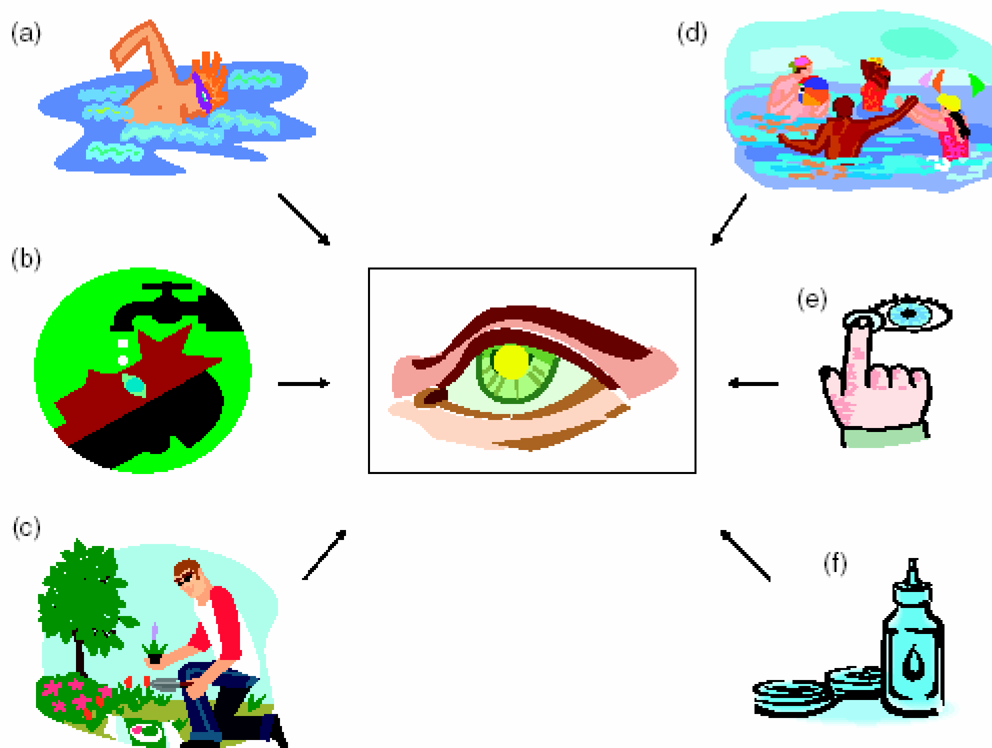


Figure 6 The risk factors contributing to *Acanthamoeba* keratitis: (a) swimming, especially while wearing contact lenses; (b) washing eyes during or immediately after contact lens wear; (c) working with soil and rubbing eyes; (d) water-related activities (splashing water), especially during or immediately after contact lens wear; (e) handling contact lenses without proper hand washing; (f) use of home-made saline (or even chlorine-based disinfectants) for contact lens cleaning. (33)

Immune responses of *Acanthamoeba* infection

The ability of *Acanthamoeba* to invade tissues depends on its ability to adhere to mucosal surfaces, migrate through tissues, and release oxygen radicals and proteases that can destroy connective tissues. In 1993, in a study of Hadas and Mazur (34) alkaline proteases of the cysteine class were found to be more active in pathogenic strains, while serine proteases were found in both pathogenic and nonpathogenic strains. Humeral immunity and complement activation are the primary mechanisms of defense against *Acanthamoeba* infection, with human serum

antibodies consisting mainly of the IgM and IgG classes. Antibodies and complement promote recognition of the amoebae by phagocytic cells. Killing of organisms by neutrophils depends primarily on lymphokines, which accelerate respiratory burst and release lysosomal enzymes that are lethal to the amoebae. The host possesses various defensive means by which it can prevent infection by free-living amoebae. When amoebae are deposited on primary sites such as skin, neuroepithelium or respiratory surfaces, only a small proportion may actually survive.

Acanthamoeba species can activate complement via the alternative pathway (35). This may be an important component of host defense against these amoebae, probably by generating opsonic factors which promote binding and damaging by neutrophils. The ability of *Acanthamoeba* to spread by the hematogenous route may in part be due to their complement activating properties. *Acanthamoeba* usually involves in the depression of host resistance (36). In 1991, Ferrante (37) established that antibodies in human serum also promote killing of *Acanthamoeba* by cytokine activated human nutrophils. In 1980, Cursons *et al*. (38) reported the human antibodies to both pathogenic and nonpathogenic *Naegleria* and *Acanthamoeba* species. Antibodies against pathogenic *Acanthamoeba* have been demonstrated in human serum, with rising anti- *Acanthamoeba* titers from 1:20 to 1:80 in New Zealanders. The antibodies were of the IgM and IgG isotypes, as IgA was rarely detected. These antibodies were shown to be capable of neutralizing cytopathogenic effects of *Acanthamoeba*e. Antibodies in human serum also promote killing of *Acanthamoeba* by cytokine activated human neutrophils.

Neutrophils: Mediated lysis phagocytic cells play an important role in immunity against pathogenic free-living amoebae. Studies have demonstrated that *Naegleria* and *Acanthamoeba* were killed by neutrophils (37). Induction of a peritoneal neutrophil-mediated inflammation in immune mice followed by an infection of *N. fowleri* into the peritoneal cavity resulted in extensive interactions of neutrophils with the amoebae. When it was examined in vitro, amoebae was adhered by a number of neutrophils and was actively lysed. Early and elegant work of Ferrante and Thong (39) demonstrated that in immune mice neutrophils surrounded, adhered to, and immobilized amoeba trophozoites, then pinched off and ingested portions of

the trophozoites, which were then ruptured. Further, in support of the central role of the neutrophils.

Macrophages and lymphocytes from human peripheral blood are not in themselves effecter cells against free-living amoebae even in the presence of antibody. For human neutrophils to kill *Acanthamoeba* effectively, several other host defense parameters must be met. Initially, neutrophils must bind to the surface of amoebae trophozoite, a fact achieved in the presence of antibody or complement components mediating neutrophils attachment. Second, neutrophils must be activated by the cytokine TNF α (Tumor Necrosis Factor alpha) derived from stimulated macrophages.

Complement mediated lysis.

Initially, non-immune host, *Acanthamoeba* species may activate the complement by alternative pathway, which leads to lysis of amoebae (36). From a conceptual point of view it is interesting to speculate that this primitive defense mechanism may have evolved concomitant with, or in response to, the equally primordial one-celled amoebae.

Although complement activation by *Acanthamoeba* may ensure though the classic antibody-mediated or the alternative pathway, there is no clear cut evidence to support complement-mediated *in vivo* lysis of amoebas. Indeed, according to Ferrante (40), it appears that the main function of complement activation is to generate opsonic factor C3b to enhance phagocytic cell recognition of and attachment to amoebas.

Laboratory diagnosis

The potential presence of *Acanthamoeba* keratitis is most commonly recognized by the presentation of free-living amoebae, using the following methods.

1. Direct observation of clinical specimens under the microscopy.
2. Staining method such as Giemsa, Field'stain (41), Gram and fluorescent antibody or Calcofluor white stain (42).
3. Culture from amoebae inoculation of filtrate on non-nutrient agar (43).

Acanthamoeba grows rapidly on 1.5% non-nutrient agar layered with gram-negative bacteria. Cyst is presented in the specimen, and *Acanthamoeba* will excyst in a favorable environment with bacterial nutrition. The trophozoite multiplies rapidly

untill the food supply are exhausted and encysted. The amoebae engulf the bacteria as it moves to the periphery of the area of bacterial growth. Usually within 2 or 3 days of incubation, the trophozoites are usually found growing outside the area of the bacterial inoculate.

One way to identify the trophozoites are to observe only one suspected trophozoite for the presence of the contractile vacuole, which will disappear and reappear after a few seconds; this feature differentiates the trophozoite from other artifacts. Within 2 or 3 more days, when the supplies of bacteria are exhausted, cyst formation will begin. When all trophozoites are encysted in an older culture, they are often arranged in clusters. The characters of cysts look like a crystal. Cysts and trophozoites can be stained for species identification or subculture on fresh overlaid *Escherichia coli* culture plates. Positive cultures should be handled in a biologic safety cabinet to avoid environmental contamination with the cyst as well as to protect health care workers.

Culture methods for *Acanthamoeba* spp.

Methods of isolation

In natural environments, *Acanthamoeba* feed on yeasts, other protozoa, bacteria, small organisms and organic particles. Any of the aforementioned can be used as growth substrates for *Acanthamoeba* in the laboratory but there are some technical problems. For example, the use of yeast and protozoa as growth substrates is problematic due to complexity in their preparations, their possible overwhelming growth and the difficulty in eradicating yeast to obtain pure axenic *Acanthamoeba* cultures. Organic substances such as glucose, proteose peptone or other substrates provide rich nutrients for unwanted organisms, *i.e.* yeasts, fungi, other protozoa and bacteria. To overcome these technical problems and to maximize the likelihood of *Acanthamoeba* isolation from environmental as well as clinical samples, protocols have been developed using simple plating assays as described below. Both of the following methods can be used to obtain large number of *Acanthamoeba* trophozoites for biochemical studies.

Isolation of *Acanthamoeba* using non-nutrient agar plates seeded with Gram-negative bacteria

This method has been used extensively in the isolation of *Acanthamoeba* from both environmental and clinical samples, worldwide. The basis of this method is the use of Gram-negative bacteria (*Escherichia coli* or *Enterobacter aerogenes*, formerly known as *Klebsiella aerogenes*, are most commonly used) that are seeded on the non-nutrient agar plate as food source for *Acanthamoeba.* The non-nutrient agar contains minimal nutrients and thus inhibits the growth of unwanted organisms. Briefly, non-nutrient agar plates containing 1% (w/v) Oxoid no.1 agar in Page's amoeba saline (PAS) (2.5mM NaCl, 1mM KH2PO4, 0.5mM Na2HPO4, 40mM CaCl2.6H2O and 20 mM MgSO4.7H2O) supplemented with 4 % (w/v) malt extract and 4 % (w/v) yeast extract are prepared, and the pH adjusted to 6.9 with KOH. Approximately 5mL of late log phase cultures of Gram-negative bacteria (*Escherichia coli* or *Enterobacter aerogenes*) are poured onto non-nutrient agar plates and left for 5 min, after which excess culture fluid is removed and plates are left to dry before their inoculation with an environmental sample or clinical specimen. Once inoculated, plates are incubated at 30°C and observed daily for the presence of *Acanthamoeba* trophozoites. Depending on the number of amoebae in the sample, trophozoites can be observed within a few hours (up to 12 h). However in the absence of amoebae, plates should be monitored for up to 7 days. Once bacteria are consumed, *Acanthamoeba* differentiate into characteristic cysts. The precise understanding of bacterial preference by *Acanthamoeba*, *i.e.* Gram-negative and Gram-positive bacteria, or why *Escherichia coli* or *Enterobacter aerogenes* are used most commonly as food substrate, and whether bacterial preferences vary between *Acanthamoeba* isolates belonging to different species/genotypes are questions for future studies.

'Axenic' cultivation of *Acanthamoeba*

Acanthamoeba can be grown 'axenically' in the absence of external live food organisms. This is typically referred to as axenic culture to indicate that no other living organisms are present. However, *Acanthamoeba* cultures may never be truly axenic as they may contain live bacteria surviving internally as endosymbionts. Under laboratory conditions, axenic growth is achieved using liquid PYG medium [proteose peptone 0.75% (w/v), yeast extract 0.75% (w/v) and glucose 1.5% (w/v)]. Briefly,

non-nutrient agar plates overlaid with bacteria are placed under UV light for 15–30 min to kill the bacterial lawn. A small piece of non-nutrient agar (stamp-sized) containing amoebic cysts is placed on plates containing these UV-killed bacteria. When amoebae begin to grow, a stamp-sized piece of the agar containing trophozoites or cysts is transferred into 10mL of sterile PYG medium containing antibiotics, *i.e.* penicillin and streptomycin. The *Acanthamoeba* switch to the PYG medium as a food source, and their multiplication can be observed within several days. Once multiplying in PYG medium, *Acanthamoeba* are typically grown aerobically in tissue-culture flasks with filter caps at 30°C in static conditions. The trophozoites adhere to the flask walls and are collected by chilling the flask for 15–30 min (at 4°C), followed by centrifugation of the medium containing the cells.

Methods of encystment

Both xenic and axenic methods have been developed to obtain *Acanthamoeba* cysts. For xenic cultures, *Acanthamoeba* are inoculated onto non-nutrient agar plates seeded with bacteria as indicated above. Plates are incubated at 30°C until the bacteria are cleared and trophozoites have transformed into cysts. Cysts can be scraped off the agar surface using phosphate-buffered saline (PBS) and used for assays. This resembles the most likely natural mode of encystment and can be effective, achieving up to 100% cysts. However, one major limitation may be the presence of bacterial contaminants that could hamper molecular and biochemical studies. For axenic encystment, *Acanthamoeba* are grown in PYG medium for 17–20 h. After this incubation, 8% glucose in RPMI 1640 (Invitrogen) is added to stimulate encystment. Plates are incubated at 30°C for up to 48 h. To confirm transformation of trophozoites into cysts, sodium dodecyl sulfate (SDS, 0.5% final concentration) is added: trophozoites are SDS-sensitive and any remaining is lysed immediately upon addition of SDS, while cysts remain intact. This method allows the simple counting of cysts using a haemocytometer and is useful in studying the process of encystment.

Comparison of the methods for detection of *Acanthamoeba* keratitis.

In 2001, Savitri *et al.* (44) described a simple procedure of Immunoperoxidase (IP) technique, using indigenously raised antibody, to screen corneal scrapings for *Acanthamoeba* cysts and trophozoites. This study sought to determine the utility of this test in the diagnosis of *Acanthamoeba* keratitis. 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% (Table 3).

In 2003, Pasricha *et al.* (45) used of 18S rRNA gene-based PCR assay for diagnosis of *Acanthamoeba* keratitis in microbial keratitis patients and compared the results 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%); however, the specificity and the positive and negative predictive values of PCR were marginally higher than the smear examination (97.8 versus 95.6%, 87.5 versus 77.8%, and 97.8 versus 97.7%) (Table 3).

Table 3 Comparison of the methods for the detection of *Acanthamoeba* keratitis

Year	Research	Author	Method	Sens (%)	Spec (%)	PPV (%)	NPV (%)	Benefit	Defect
2001	Evaluation of immunoperoxidase staining technique in the diagnosis of *Acanthamoeba* keratitis	Savitri *et al*	Immunope-roxidase technique	100	94	80	100	high sensitivity	high cost, difficult
			Culture	83	100	100	94	easy, inexpensive	require familiarity with the morphology, use long time
2003	Use of 18S rRNA Gene-Base PCR Assay for Diagnosis of *Acanthamoeba* keratitis in Non-Contact Lens Wearers in India	Pasricha *et al*	PCR	87.5	97.8	87.5	97.8	high sensitivity and specificity	high cost, difficult
			Smear (Calcofluor-white, Gram stain, Giemsa stain)	87.5	95.6	77.8	97.7	easy, inexpensive	require familiarity with the morphology ,low sensitivity
2006	Development of a real-time PCR assay for quantification of *Acanthamoeba* trophozoites and cysts	Riviere *et al*	real-time PCR	1 pg -10 ng	-	-	-	Sensitive, rapid, reduced contamination	high cost, difficult

Abbreviations: PPV, Positive Predictive Value; NPV, Negative Predictive Value.

Treatment

Acanthamoeba infections are highly resistant to chemotherapeutic agents, especially in the encysted stage; and mostly disappointing clinical treatment had been reported. Good therapeutic results may depend on the time of diagnosis, the virulence of the organism and the eventual acquisition of resistance by the pathogen. Corticosteroids are necessary both to control active inflammation and to permit later successful keratoplasty. With timely diagnosis and medical treatment with a combination of antiamoebic drugs and avoidance of topical corticosteroids, most cases of *Acanthamoeba* keratitis can be cured, with an excellent prognosis for visual recovery.

At present, the great majority of infections occur in wearers of contact lens on a few occasions in both eyes. Since amoebae have been cultured from all types of lenses solution utilized, good hygienic care is important. Only sterilized, commercially prepared products should be changed frequently, *Acanthamoeba* are resistant to freezing, drying, various antimicrobial agents, and the usual levels of chlorine used in drinking water and in swimming pools. Cold chemical disinfection of lens was inferior to heat sterilization. Fortunately, standard methods for the sterilization of surgical instruments are effective (46).

Review of ophthalmic reports showed several series of medical cures with specific combination therapies including 0.1% propamidine isethionate, 0.15% dibromopropamidine isethionate, neomycin-polymyxin B-gramicidin, 1% miconazole nitrate and corticosteroid eye drops. In 1985, Wright *et al.*(47) reported the first medical cure of *Acanthamoeba* keratitis using a combination of topical 0.15% dibromopropamidine, 0.1% propamidine isethionate, neomycin and 1% prednisolone acetate over a one-year period. *Acanthamoeba* organisms were recovered from the cornea and therapy was initiated at a relatively late stage (two months) in the disease. In 1990, Ishibashi *et al.* (48) reported three cases of successful medical management of *Acanthamoeba* keratitis using a combination of oral itraconazole, topical miconazole, and debridement. In all cases, anti-amoebic therapy was initiated within nine weeks or less from the onset of the patient symptoms.

By preventing the free transformation of the two existing forms of *Acanthamoeba*, corticosteroids may theoretically permit amoebicidal agents to destroy the trophozoites, whereas the host response clears the inactive amoebic cysts. In clinical *Acanthamoeba* infections, there is no report on the effect of corticosteroids on the keratitis, although in many cases, the clinical symptom was improved and pain was less (or disappeared) after the addition of topical corticosteroids. However, there are examples of clinical deterioration and relapse of keratitis during corticoseroids in treatment of *Acanthamoeba* keratitis (10).

The most common misdiagnosis, resulting in the delay of appropriate antiamoebic therapy was herpes simplex keratitis, having been diagnosed initially in all patients. The range of initial signs in the patients included punctuates, corneal epithelial discrifrom, stromal edema, perineural infiltrate and central or paracentral stromal infiltrates.

Bacterial endosymbionts

The presence of endosymbionts has been demonstrated in many protozoan species. The symbionts may either occur 'naturally' or may represent more recently phagocytosed organisms that have adapted to the intracellular environments. Phagocytosed bacteria may grow and reproduce within the protozoan host and, in some cases, have been shown to eventually become symbionts (49). The process itself and its pathogenic significance is not well known. Hardly any data can be found on the mechanism by which it develops and by which it supports or at least allows the survival of both organisms. Small free-living amoebae such as *Naegleria* and *Acanthamoeba* have been found to have bacterial endosymbionts. In 1995, Gauton and Fritsche (50) have suggested that endosymbionts occurs commonly among members of the family *Acanthamoebidae*. Since the first report by Rowbotham (28) in 1980, the association of the amoebae *Naegleria* and *Acanthamoeba* with the symbiont *Legionella pneumophilla*, the causative agent of *Legionnaires's* disease has been a subject of interest. *Acanthamoeba*, *Naegleria*, *Hartmannella*, *Vahlkampfia* and *Echinamoeba* have been shown to support the growth of *Legionella* (51) and environmental growth of *Legionella* in the absence of protozoa has not been documented. It is though likely that the protozoa

are the primary means of proliferations of these bacteria under natural conditions. This inter-relationship within the ecosystem can modify the virulence of *Legionella* can be viable and detectable by cultivation on BCYE (Buffered Charcoal Yeast Extract) agar-based systems (52). In 1994, Hay (53) have proposed that the later observation may have profound implications with regard to surveillance of water systems for *Legionella*; especially, with respect to prevention of outbreaks of nosocomial Legionnaires' disease.

The ability of *Legionellae* to survive as intracellular parasites of both protozoan and mammalian cells may be unprecedented. However, a number of bacteria have been reported to survive as parasites of endosymbionts of free-living protozoa and some of these bacteria are also potential pathogens of vertebrates. Scientist had theorized that some intracellular pathogens of higher vertebrates could have acquired the ability to infect by first adapting to intracellular life in protozoa. Predation by free-living protozoa appears to be an ideal selective pressure for the evolution of these intracellular bacteria. It is tempting to imagine that they subsequently acquired mechanisms for infecting higher eukaryotic cells. However, this theory contradicts a central doctrine of evolution that more highly evolved organisms tend to become more specialized. This matter can be examined further by considering the host range of *Legionella.*

Table 4 Protozoa supporting the growth of *Legionellae* (52).

Category	Organism
Amoeba	*Acanthamoeba castellanii* *Acanthamoeba polyphaga* *Acanthamoeba palestinensis* *Acanthamoeba royreba* *Acanthamoeba culbertsoni* *Naegleria gruberi* *Naegleria fowleri* *Naegleria lovaniensis* *Naegleria jadini* *Hartmannella vermiformis* *Hartmannella cantabrigiensis* *Vahlkampfia jugosa*
Ciliated protozoa	*Tetrahymena pyriformis*

Molecular biology study of *Acanthamoeba* spp.

The polymerase chain reaction (PCR) is a powerful and widely used technique that has greatly advanced in genetic analysis. PCR allows specific DNA sequences, usually corresponding to genes or parts of genes to be copied from chromosomal DNA in a simple enzyme reaction. The only requirement is some of the DNA sequences at either end of the region to be copied are known. DNA corresponding to the sequence of interest is copied or amplified by PCR more than a million folds and becomes the predominant DNA molecule in the reaction. Therefore, sufficient DNA is obtained for detailed analysis or manipulation of the amplified gene (54). The polymerase chain reaction has recently been modified to type organisms at the species level. Some approaches required preexisting sequence data, while other have shown that even arbitrary primers can differentiate a group of organisms by generating banding patterns similar to those in RFLP (restriction fragment length polymophism) analysis (55). The basic classification of *Acanthamoeba* spp. is based on the morphological characteristics of trophozoites and cysts which subdivided the member of this genus into three morphological groups. However, to classify the amoebae at species level and strains need to be specified by molecular characteristics such as analysis of isoenzyme profiles (56), RFLP (restriction fragment length

polymophism) patterns of mitochondrial DNA, or nuclear DNA (25) or 18S rRNA gene (57).

In 1985, Costas and Griffith (58) reported the application of starch gel electrophoresis for differentiation of 32 strains of *Acanthamoeba* from the same isolation. Strains that had been assigned to the same species especially *Acanthamoeba castellanii* and *Acanthamoeba polyphaga* did not always have similar enzyme patterns. In 1983, De Jonckheere (56) demonstrated the isoenzyme analysis by agarose isoelectric focusing for the taxonomy of the genus *Acanthamoeba* and also revealed a large degree of interstrain genetic diversity by mitochondrial DNA restriction fragment length polymophisms (mtRFLP) (59). Isoenzyme patterns and mt-RFLP analysis have highlighted ambiguities in the morphological classification scheme, but not directly addressed the phylogeny of *Acanthamoeba*.

In 1990, Johnson *et al*. (60) analyzed partial nuclear 18S rRNA sequences from seven isolates of *Acanthamoeba* and obtained results that were concordant with the classification in three morphological groups. In 1996, Gast *et al*. (61) investigated 18S rRNA gene phylogeny using 18 isolates of *Acanthamoeba* from morphological group II and group III. These groups were chosen because they contained species most frequently isolated from human infections. The amount of sequence dissimilarity among the 18 isolates ranged from 0-11.9% and subdivided into four rDNA sequence types. Fifteen of the isolates formed a tight phylogenetic cluster designated sequences type T4. In 1998, Stothard *et al*. (57) examined 18S rRNA sequence variation in a group of 53 strains including the original 18 isolates plus 35 new isolates. Sixteen species and all the morphological groups were represented in their study. Eight more sequence types were identified. They found that morphological group I isolates were so highly divergent and probably contained one or more unique genera presenting T7, T8 and T9. Group II which included major population of *Acanthamoeba* in nature contained T3, T4 and T11 while group III contained more different 18S rRNA sequence types including T1, T2, T5, T6,T10 and T12 (57).

In Thailand, Kaewjai (62) detected *Acanthamoeba* by culture method and to genotype individual isolates using PCR and PCR-RFLP of the 18S rRNA gene. A pair of genus-specific primers was designed based on the 18S rRNA of *A. castellanii*,

which amplified approximately 2.1 kb amplicon, followed by digestion with 3 restriction endonucleases, i.e *Hae*III, *Hinf*I and *Msp*I, were analyzed and compared with the reference acanthamoebae. For *Acanthamoeba* speciation in this study, use of PCR-RFLP product derived from the 18S rRNA gene after *Msp*I digestion was superior.

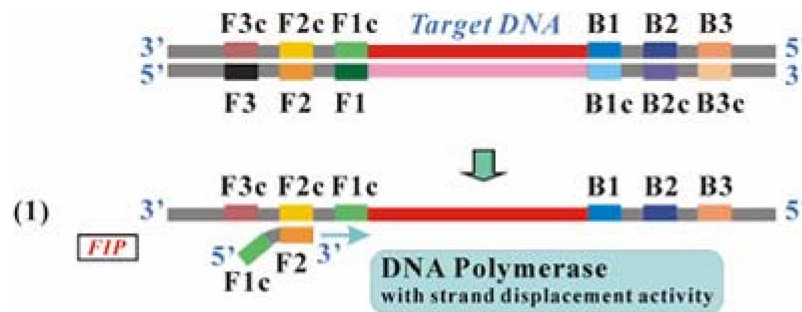
Most studies in molecular genetics involve the used of PCR at some stage, normally as part of an overall strategy and in association with other techniques. For example, DNA amplified by PCR can be used for DNA sequencing as a probe in Northern and Southern blotting, and to generate clones. PCR has applications in most areas of biology and medicine as well as in unexpected subjects such as anthropology and archaeology. It is also an important tool in the biotechnology industries. Advantage of the sensitivity and specificity of PCR to generate data may be useful for clinical, ecological or taxonomic studies of *Acanthamoeba.*

Loop-mediated isothermal amplification of DNA (LAMP)

A method recently developed by Notomi *et al.* (63) in 2000, relies on autocycling strand displacement DNA synthesis by a *Bst* DNA polymerase. LAMP requires two specially designed inner and two outer primers as such; LAMP amplifies DNA with high specificity, efficiency, and rapidity under isothermal conditions. Since the LAMP reaction is done under isothermal conditions (63 to 65°C), simple incubators, such as a water bath or block heater, are sufficient for the DNA amplification. Moreover, LAMP synthesizes 10 to 20 µg of target DNA within 30 to 60 min, and the LAMP reaction appears to be limited only by amount of deoxynucleoside triphosphates and primers. In the process, a large amount of pyrophosphate ion is produced, which reacts with magnesium ions in the reaction to form magnesium pyrophosphate, a white precipitate by product. This phenomenon allows easy and rapid visual identification that the target DNA was amplified by LAMP. Therefore, LAMP is a highly sensitive and specific DNA amplification technique suitable for diagnosis of an infectious disease both in well equipped laboratories and in field situations.

The principle of LAMP method

When the target gene (DNA template as example) and the reagents are incubated at a constant temperature between 60-65°C, the following reaction steps proceed.



STEP 1: As double stranded DNA is in the condition of dynamic equilibrium at the temperature around 65°C, one of the LAMP primers can anneal to the complimentary sequence of double stranded target DNA, then initiates DNA synthesis using the DNA polymerase with strand displacement activity, displacing and releasing a single stranded DNA. With the LAMP method, unlike with PCR, there is no need for heat denaturation of the double stranded DNA into a single strand. The following amplification mechanism explains from when the FIP anneals to such released single stranded template DNA.

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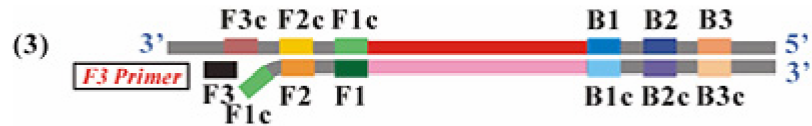
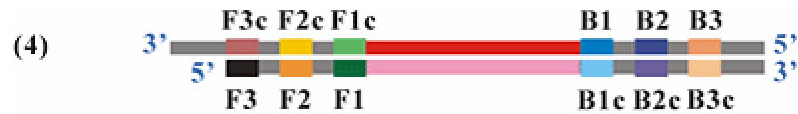
STEP 2: Through the activity of DNA polymerase with strand displacement activity, a DNA strand complementary to the template DNA is synthesized, starting from the 3' end of the F2 region of the FIP.

Figure 7 The principle of LAMP method

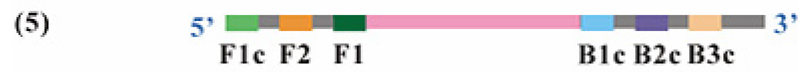
Source: http://www.loopamp.eiken.co.jp/e/lamp/anim.htm [Accessed Nov. 11, 2006]



STEP 3: The F3 Primer anneals to the F3c region, outside of FIP, on the target DNA and initiates strand displacement DNA synthesis, releasing the FIP-linked complementary strand.



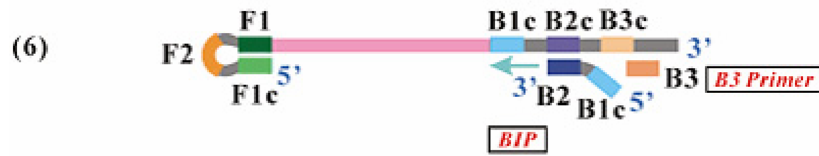
STEP 4: A double strand is formed from the DNA strand synthesized from the F3 Primer and the template DNA strand.



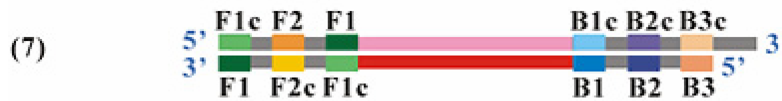
STEP 5: The FIP-linked complementary strand is released as a single strand because of the displacement by the DNA strand synthesized from the F3 Primer. Then, this released single strand forms a stem-loop structure at the 5' end because of the complementary F1c and F1 regions.

Figure 7 The principle of LAMP method (cont.)

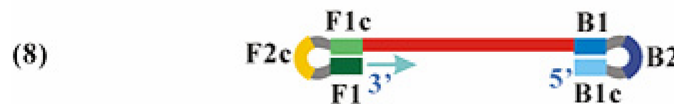
Source: http://www.loopamp.eiken.co.jp/e/lamp/anim.htm [Accessed Nov. 11, 2006]



STEP 6: This single strand DNA in Step (5) serves as a template for BIP-initiated DNA synthesis and subsequent B3-primed strand displacement DNA synthesis. The BIP anneals to the DNA strand produced in Step (5). Starting from the 3' end of the BIP, synthesis of complementary DNA takes place. Through this process, the DNA reverts from a loop structure into a linear structure. The B3 Primer anneals to the outside of the BIP and then, through the activity of the DNA polymerase and starting at the 3' end, the DNA synthesized from the BIP is displaced and released as a single strand before DNA synthesis from the B3 Primer.



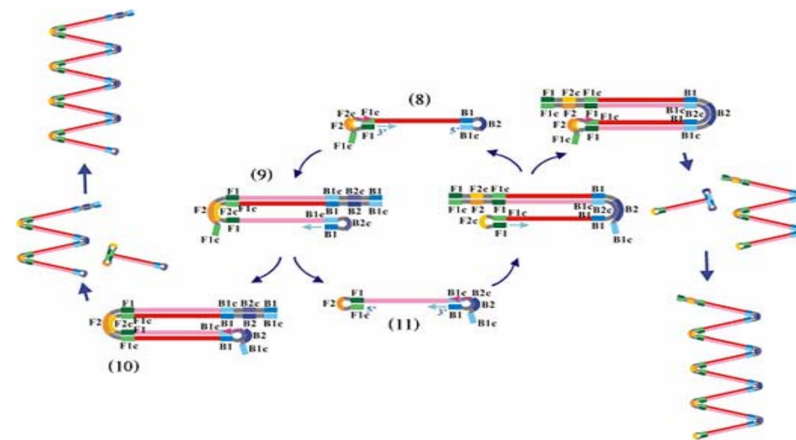
STEP 7: Double stranded DNA is produced through the processes described in Step (6).



STEP 8: The BIP-linked complementary strand displaced in Step (6) forms a structure with stem-loops at each end, which looks like a dumbbell structure. This structure serves as the starting structure for the amplification cycle in the LAMP method (LAMP cycling). The above process can be understood as producing the starting structure for LAMP cycling.

Figure 7 The principle of LAMP method (cont.)

Source: http://www.loopamp.eiken.co.jp/e/lamp/anim.htm [Accessed Nov. 11, 2006]



Basic principle (8) - (11) (Cycling amplification step): A dumbbell-like DNA structure is quickly converted into a stem-loop DNA by self-primed DNA synthesis. FIP anneals to the single stranded region in the stem-loop DNA and primes strand displacement DNA synthesis, releasing the previously synthesized strand. This released single strand forms a stem-loop structure at the 3' end because of complementary B1c and B1 regions. Then, starting from the 3' end of the B1 region, DNA synthesis starts using self-structure as a template, and releases FIP-linked complementary strand (Step (9)). The released single strand then forms a dumbbell-like structure as both ends have complementary F1 - F1c and B1c - B1 regions, respectively (Step (11)). This structure is the 'turn over' structure of the structure formed in Step (8). Similar to the Steps from (8) to (11), structure in Step (11) leads to self-primed DNA synthesis starting from the 3' end of the B1 region. Furthermore, BIP anneals to the B2c region and primes strand displacement DNA synthesis, releasing the B1-primed DNA strand. Accordingly, similar structures to Steps (9) and (10) as well as the same structure as Step (8) are produced. With the structure produced in Step (10), the BIP anneals to the single strand B2c region, and DNA synthesis continues by displacing double stranded DNA sequence. As a result of this process, various sized structures consisting of alternately inverted repeats of the target sequence on the same strand are formed.

Figure 7 The principle of LAMP method (cont.)

Source: http://www.loopamp.eiken.co.jp/e/lamp/anim.htm [Accessed Nov. 11, 2006]

In 2003, Kuboki *et al.* (64) used LAMP for detection of parasites in *the Trypanosoma brucei* group (including *T. blucei blucei, T. blucei gambiense, T. blucei rhodesiense*, *and T.enansi*) and *T. congolense*. The results show that the sensitivity of the LAMP-based method for detection of trypanosomes in vitro is up to 100 times higher than that of PCR-based methods. In vivo studies in mice infected with human-infective *T.blucei gambiense* further highlight the potential clinical importance of LAMP as a diagnostic tool for the identification of African trypanosomiasis.

In 2004, Ikadai *et al.* (65) compared of the PCR and LAMP methods with microscopic examination for the detection of *Babisia gibsoni* infections in blood samples from 945 field dogs in Aomori Prefecture and 137 field dogs in Okinawa Prefecture showed that 37 and 15 dogs, respectively, were positive by the PCR and LAMP methods and that 16 and 12 dogs, respectively, were positive by light microscopic examination. All samples found to be positive by microscopic examination were also positive by the PCR and LAMP methods. The results of the PCR and LAMP methods agreed for samples with positive results by either method. Moreover, nonspecific reactions were not observed by the LAMP method. These results suggest that the LAMP method provides a useful tool for the detection of *B.gibsoni* infections in dogs.

In 2006, Poon *et al.* (10) used LAMP for detetion of *Plasmodium falciparum* DNA directly from Heat-Treated Blood. Results were interpreted by a turbidity meter in real time or visually at the end of the assay. To evaluate the assay, DNA from these samples was purified and tested by PCR. Results from the LAMP and PCR assays were compared, the sensitivity and specificity of the LAMP assay were 95% and 99% respectively, and its does not require purified DNA for efficient DNA amplification, thereby reducing the cost and turnaround time for *P. falciparum* diagnosis. The assay requires only basic instruments, and assay positivity can be verified by visual inspection.

In 2006, Alhassan *et al.* (66) used LAMP for detetion of *Theileria equi* and *Babesia caballi* infections. The primer sets amplified *T. equi* and *B. caballi* up to 10^{-6} dilution of 10-fold serially diluted samples. Furthermore, DNA extracted from blood collected from a horse experimentally infected with *T. equi* was amplified by a *T. equi* LAMP primer set from days 2 to 35 post-infection, demonstrating the high sensitivity of these primers. Of 55 samples collected from China, 81.8% and 56.3% were positively detected by LAMP for *T. equi* and *B. caballi* infections, respectively. In contrast, 91.8%

and 45.9% of the 37 samples collected from South Africa were LAMP positive for *T. equi* and *B. caballi*, respectively. These results suggest that LAMP could be a potential diagnostic tool for epidemiological studies of equine piroplasmosis.

In 2007, Han *et al.* (67) used LAMP for the detection of four species of human malaria parasites, *Plasmodium falciparum*, *P.vivax*, *P.malariae*, and *P.ovale*. They evaluated the sensitivity and specificity of LAMP in comparison with microscopic examination and nested PCR. LAMP showed a detection limit (analytical sensitivity) of 10 copies of the target 18S rRNA genes for *P.malariae*, and *P.ovale*, and 100 copies for genus *Plasmodium* , *P. falciparum*, and *P.vivax*. LAMP detected malaria parasites in 67 out of 68 microscopically positive blood sample (98.5% sensitivity), and in 3 out of 53 microscopically negative samples (94.3% specificity), in good agreement with the results of nested PCR. LAMP reactions yielded results within about 26 min on average for detection of genus *Plasmodium*, 32 min for *P. falciparum*, 31 min for *P. vivax*, 35 min for *P. malariae*, and 36 min for *P. ovale*. Accordingly, LAMP, compared to microscopy, had similar sensitivity and greater specificity, and compared to nested PCR it yielded similar results in shorter turnaround time. Because it can be performed with simple technology, i.e. heat-treated blood as a template, reaction in a water- bath, and inspection of the results by the naked eye using fluorescent dye, LAMP may provide a simple and reliable test for routine screening of malaria parasites in both clinical laboratories and malaria clinics in endemic areas.

Real-Time PCR assay.

Real-Time PCR monitors the fluorescence emitted during the reaction as an indicator of amplicon production during each PCR cycle as opposed to the endpoint detection. Real-Time chemistries allow for the detection of PCR amplification during the early phases of the reaction. Measuring the kinetics of the reaction in the early phases of PCR provides a distinct advantage over traditional PCR detection. Traditional methods use Agarose gels for detection of PCR amplification at the final phase or end-point of the PCR reaction.

Real-Time reporter

The real-time PCR system is based on the detection and quantitation of a fluorescent reporter (68). This signal increases in direct proportion to the amount of PCR

product in a reaction. By recording the amount of fluorescence emission at each cycle, it is possible to monitor the PCR reaction during exponential phase where the first significant increase in the amount of PCR product correlates to the initial amount of target template. The most commonly used flurogenic oligoprobes rely upon fluorescene resonance energy transfer (FRET) between fluorogenic labels or between one fluorophore and a dark or black- hole non- fluorescent quencher (NFQ), which disperses energy as heat rather than fluorescence. FRET is a spectroscopic process by which energy is passed between molecules separated by 10-100 A° that have overlapping emission and absorption spectra. There are three main fluorescene-monitoring systems for DNA amplification (1) hybridizing probes; (2)hydrolysis probes include Taqman probes, molecular beacons and scorpions; (3) DNA-binding agents.

DNA-binding agents

The cheaper alternative is the double-stranded DNA binding dye chemistry, which quantitates the amplicon production (including non- specific amplification and primer dimer-complex) by the use of a non-sequence specific fluorescent intercalating agent (SYBR-green I or ethidium bromide). It does not bind to ssDNA. SYBR green is a fluorogenic minor groove binding dye that exhibits little fluorescence when in solution but emits a strong fluorescent signal upon binding to double-stranded DNA. Disadvantages of SYBR green-based real-time PCR include the requirement for extensive optimisation. Furthermore, non-specific amplifications require follow-up assays (melting point of dissociation curve analysis) for amplicon identification (69). The system of DNA-binding dyes (SYBR Green) as shown in Figure 8.

Real-Time PCR Quantitation

Reactions are characterized by the point in time during cycling when amplification of a PCR product is first detected rather than the amount of PCR product accumulated after a fixed number of cycles. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed. An amplification plot is the plot of fluorescence signal versus cycle number. In the initial cycles of PCR, there is little change in fluorescence signal. This defines the baseline for the amplification plot. An increase in fluorescence above the baseline indicates the detection of accumulated PCR product. A fixed fluorescence threshold can be set above the baseline. The parameter CT (threshold cycle) is defined as the fractional cycle number at which the fluorescence passes the fixed

threshold. A plot of the log of initial target copy number for a set of standards versus CT is a straight line. Quantification of the amount of target in unknown samples is accomplished by measuring CT and using the standard curve to determine starting copy number (70).

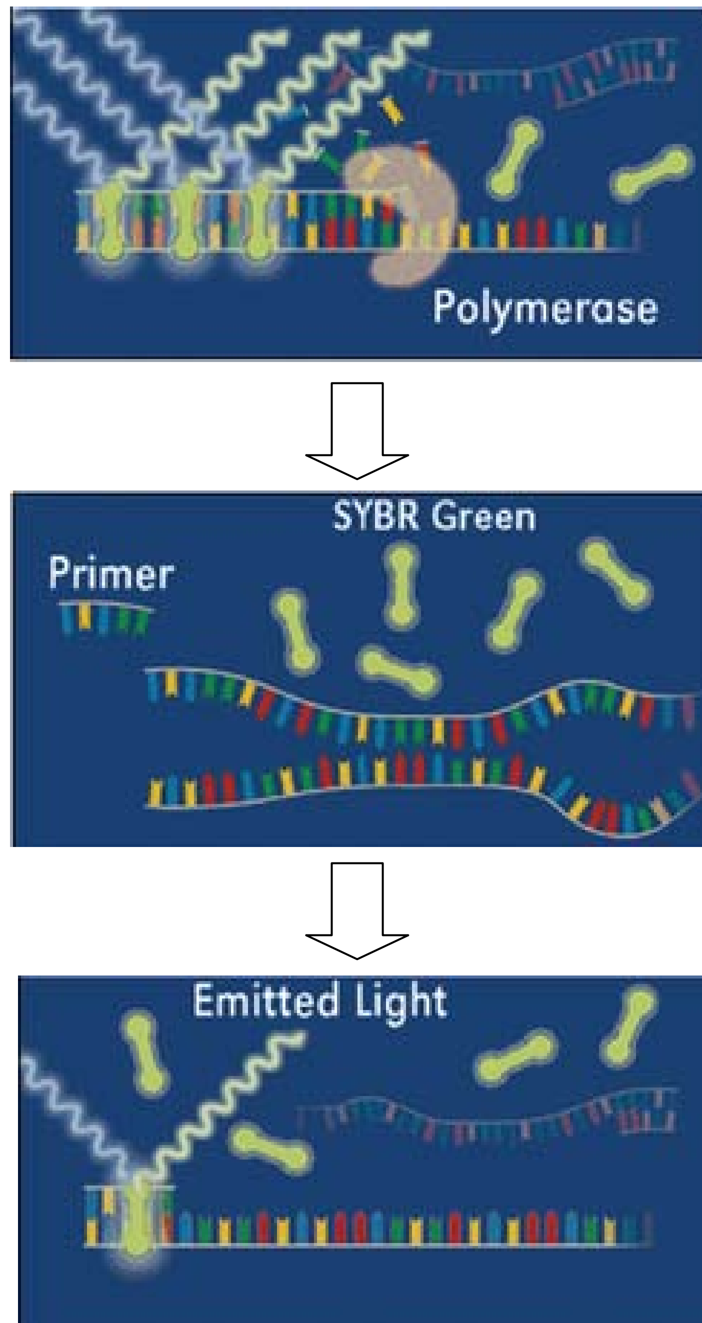


Figure 8 The principle of Real-Time PCR (SYBR) assay

Source: http://gene-quantification.de/chemistry.html#sybrgreen

Advantage and disadvantage

Real-time chemistries allow for the detection of PCR amplification during the early phases of the reaction. Measuring the kinetics of the reaction in the early phases of provides a distinct advantage over traditional PCR detection. Traditional methods use Agarose gels for detection of PCR amplification at the final phase or end-point of the PCR reaction. These techniques are cumbersome, time consuming and require multiple manipulations of the samples, thus increasing the risk of carry-over contamination.

In 2004, Riviere *et al.* (71) used Real-time PCR assay based on a TaqMan probe that hybridizes onto 18S rDNA. This probe is specific to the *Acanthamoeba* genus. The assay was successful with both the trophozoite and the cyst forms of *Acanthamoeba*. highly sensitive, it proved to permit detection of fewer than 10 cells, even those that are not easily cultivable, such as the cyst forms.

CHAPTER III
MATERIALS AND METHODS

This study was experimental designed, which LAMP method and cultivation method were compared. Real-time PCR was a confirmed method. The materials and methods were performed and prepared as following.

Study area and population

Study area

The study was conducted at Silapakorn University, Nakhornpathom Province. Laboratories were performed at the Faculty of Public Health, Department of Parasitology, Mahidol University, Bangkok.

Study population

1. All sexes and ages 15-40 years.
2. 150 students of Silapakorn University, Nakhornpathom Province who used contact lens
3. Written consent form as appropriate

Sample size calculation

In Thailand, Roongruangchai and Supadirekkul (7) reported 2.4 % (2/87) prevalence of *Acanthamoeba* spp. in contact lens cases.

$$n = \frac{[Z^2_{\alpha/2} PQ]}{(d^2)}$$

P = proportion or prevalence of diseases in population (0.024)

Q = 1-P (0.976)

d = interval confidence of 97 % (0.03)

$Z^2_{\alpha/2}$ = standard score of interval confidence is $1-\propto$ $(1.96)^2$

$$n = \frac{(1.96)^2(0.024)(0.976)}{(0.03)^2}$$

Total sample = 100.

The prevalence of this infection is very rare, so the number of sample collection was 50 added.

Study materials, and research tools

1. Laboratory equipment and tools

1.1 DNA extraction kit

1.2 Centrifuge

1.3 Water bath

1.4 Inverted Microscopy

1.5 Incubator

1.6 Block heater

2. Recorded form

2.1 Test Record form (Appendix B)

***Acanthamoeba* spp.** *Acanthamoeba* spp. was kindly provided by Asst Prof. Kosol Roongruangchai, Faculty of Medicine, Siriraj Hospital, Mahidol University.

***Escherichia coli* culture** Bacterial culture was grown in TSB medium, the culture was washed with Page's amoeba saline and inactivated at 60°C for 15 min before use. The turbidity of culture suspention was adjusted to be equal to 0.5 McFarland standard (approximately 10^8 CFU/ mL).

Study methods

1. When the contact lens storage cases were collected, each of them was sealed in plastic bag and carried to the laboratory for tested.
2. The contact lens storage cases were opened under aseptic conditions then the amoeba salines were dropped into the contact lens storage cases.
3. A sterile cotton bud was scraped over the internal surface of the contact lens storage cases.
4. The solution in the contact lens storage cases was sediment by centrifugation at 2,000 x g, for 5 min. The supernatant were discarded. The pellet were obtained and then subjected to culture and DNA extraction. The pellets were stored in microcentrifuge tube at –20°C until use.

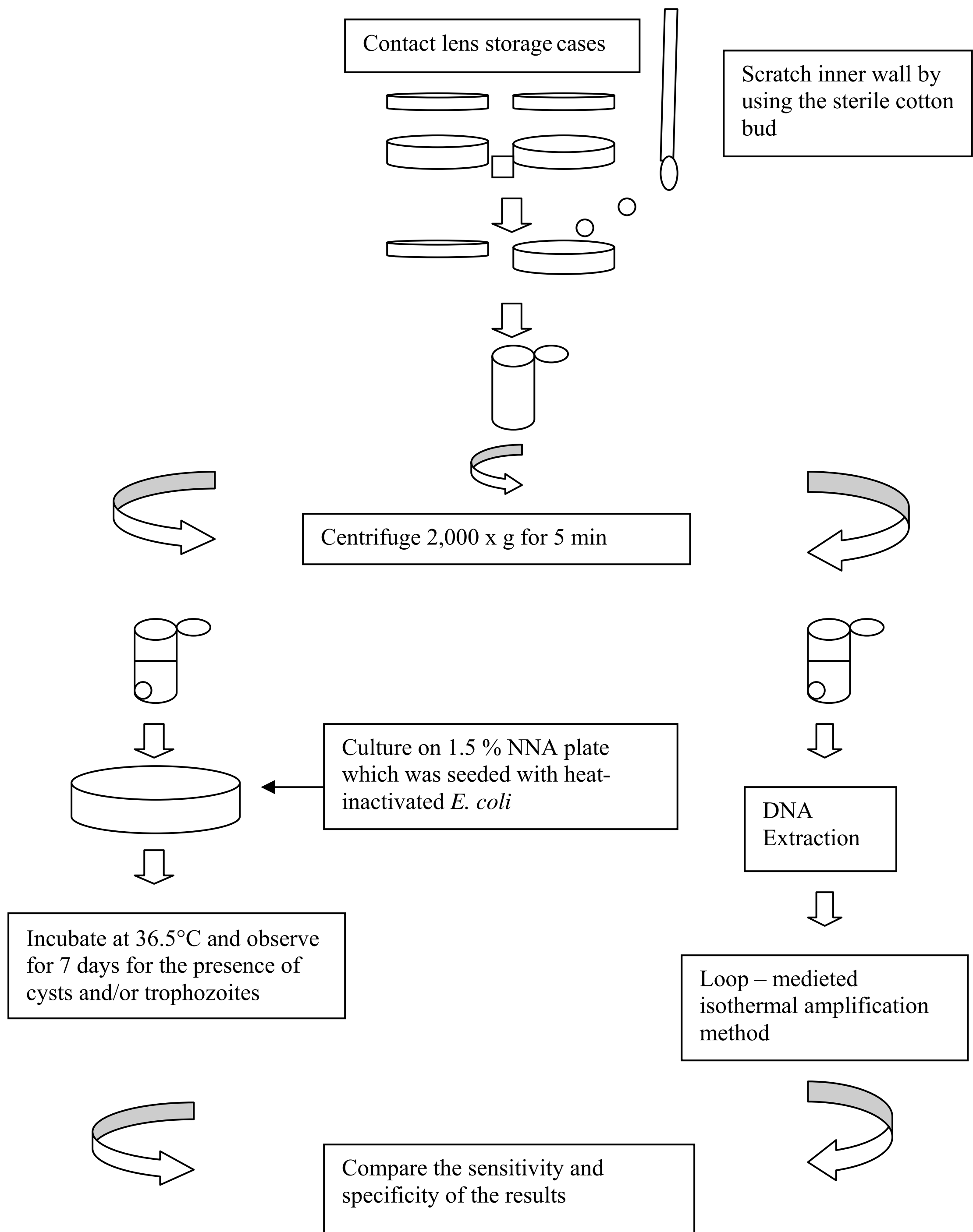


Figure 9 Conceptual Framework

Observation of the temperature for *Acanthamoeba* spp. growth in contact lens storage cases.

For observation of the temperature for *Acanthamoeba* spp. growth in contact lens storage cases. In this study, we performed artificial infection of cysts of *Acanthamoeba* spp. kindly provided by Asst Prof. Kosol Roongruangchai, Faculty of Medicine, Siriraj Hospital, Mahidol University to the contact lens storage cases.

The artificial infection of *Acanthamoeba* spp. to contact lens storage cases.

1. The 50 µl of cysts of *Acanthamoeba* spp. were dropped onto the contact lens storage cases containing contact lens solution and 50 µl of *E.coli.*
2. Incubated at room temperature (36.8-37.2°C) and observed for the presence of cysts and/or trophozoites for 1weeks.

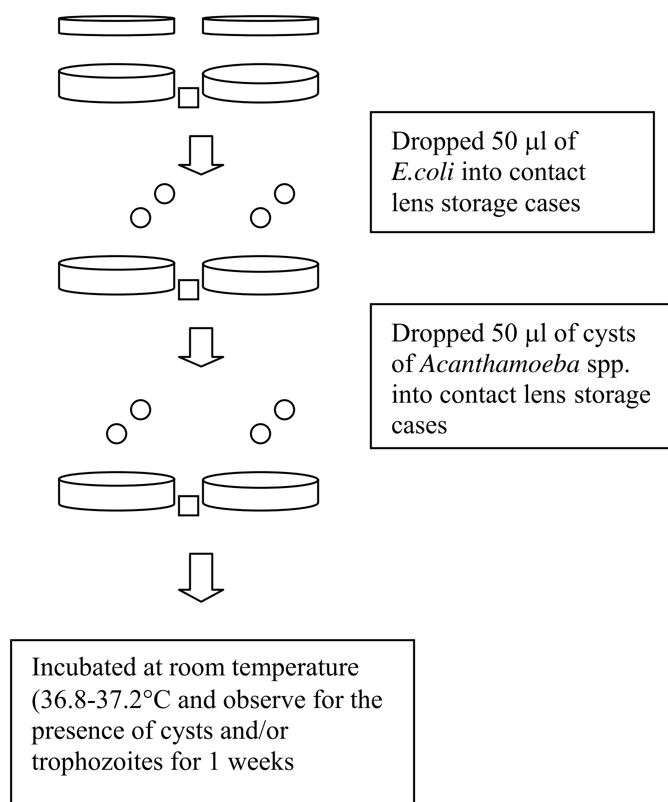


Figure 10 Methodology flow chart of the artificial infection of *Acanthamoeba* spp. to contact lens storage cases.

The cultivation of *Acanthamoeba* on non-nutrient agar

1. The pellets from the centrifuged solution in the contact lense storage cases were aseptically dropped onto 1.5 % non-nutrient agar plates which were seeded with heat-inactivated *E. coli*.
2. Incubated at 36.5°C and observed for 7 days for the presence of cysts and/ or trophozoites under an inverted microscope. The trophozoites were usually found growing away from the area of the bacterial inoculation.
3. Cyst of each group has been morphologically described as the follow:

Astronyxids: species having relatively large cysts with smooth ectocyst and stellate endocyst.

Polyphagids: species having wrinkled ectocyst and endocyst can be stellate, polygonal, triangular, or oval.

Culbertsonids: species typically having thin smooth ectocyst with round endocyst.

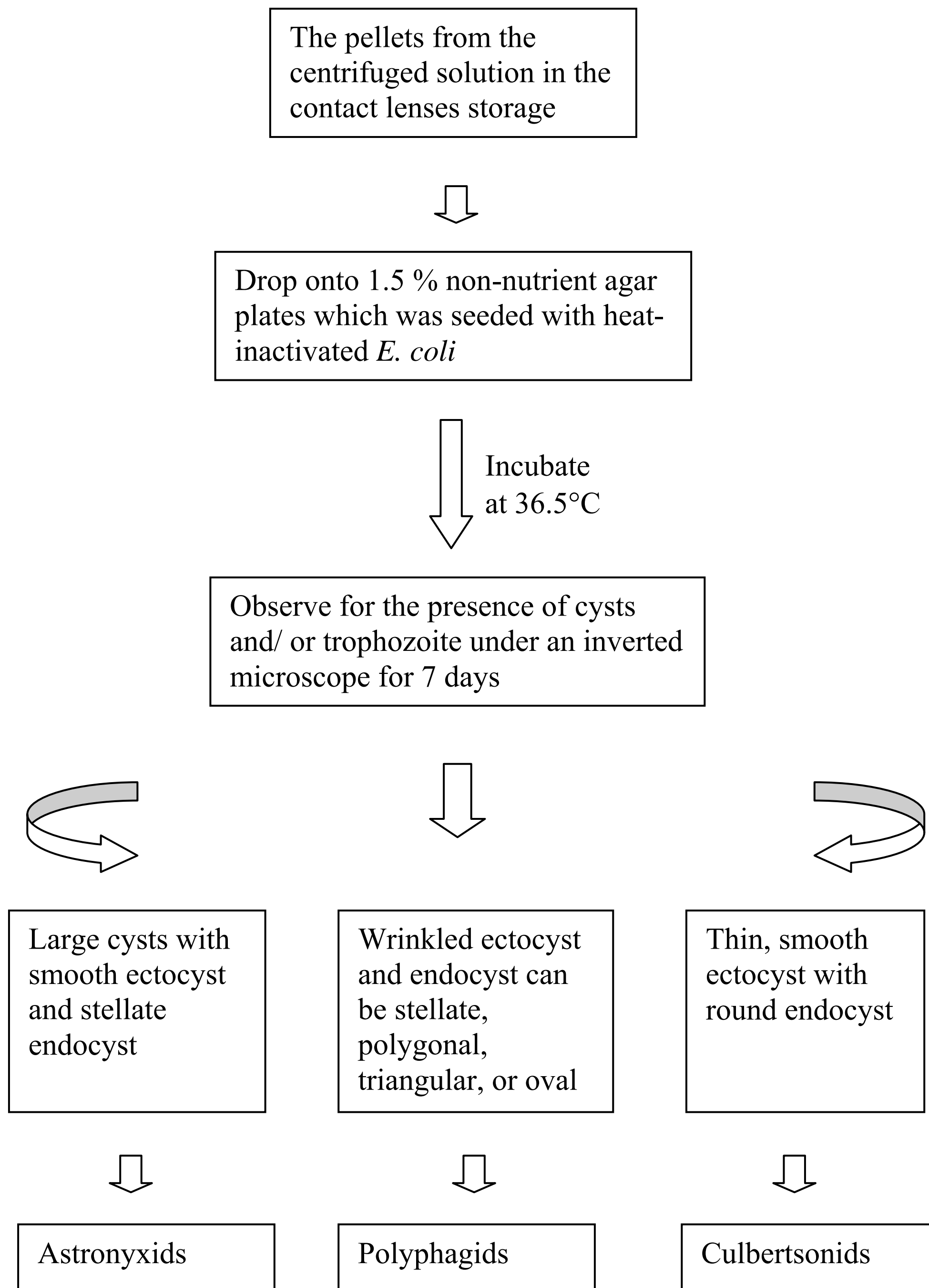


Figure 11 Methodology flow chart of culture technique

DNA extraction

Acanthamoeba cysts were resistant to the ordinary cell lysis buffer in the DNA extraction process which could be observed under microscopic examination. In this study, we try to prepare *Acanthamoeba* cysts for DNA extraction by repeated freezing at -20°C for 1hr and thawing at room temperature then observed the lysis of *Acanthamoeba* cyst walls under microscopic examination.

DNA extraction procedure

Nucleospin tissue extraction kit imported by Pacific Science Company was used according to the protocol instruction.

1. The Pre-lysis is the first step, the pellet was resuspended in 180 µl buffer T1 by pipetting up and down.
2. 25 µl of proteinase K was added.
3. The suspension was vortexed vigorously and was incubated at 56°C until cell completely lysed (at least 30 minute.).
4. 200 µl of buffer B3 was added, vortexed vigorously and incubated at 70°C for 10 minute.
5. Centrifuge for 5 minutes at 11,000 × g, and the supernatant was transferred to a new microcentrifuge tube.
6. 210 µl ethanol (100%) was added to the sample and vortex vigorously.
7. For each sample, place one Nucleospin tissue column into a 2 ml collecting tube.
8. The sample was applied to the column and centrifuged for 1 minute at 11,000 x g. The flow-through was discarded.
9. The silica membrane was washed twice with 500 µl of buffer BW and 600 µl of buffer B5, respectively at 11,000 x g, 1 minute. Next, the column was centrifuged for 1 minute at 11,000 x g to remove the residual ethanol.
10. DNA were eluted with 100 µl pre-warm elution buffer BE (70°C) and spun at 11,000 x g for 1 minute.
11. DNA was stored at -20°C until use.

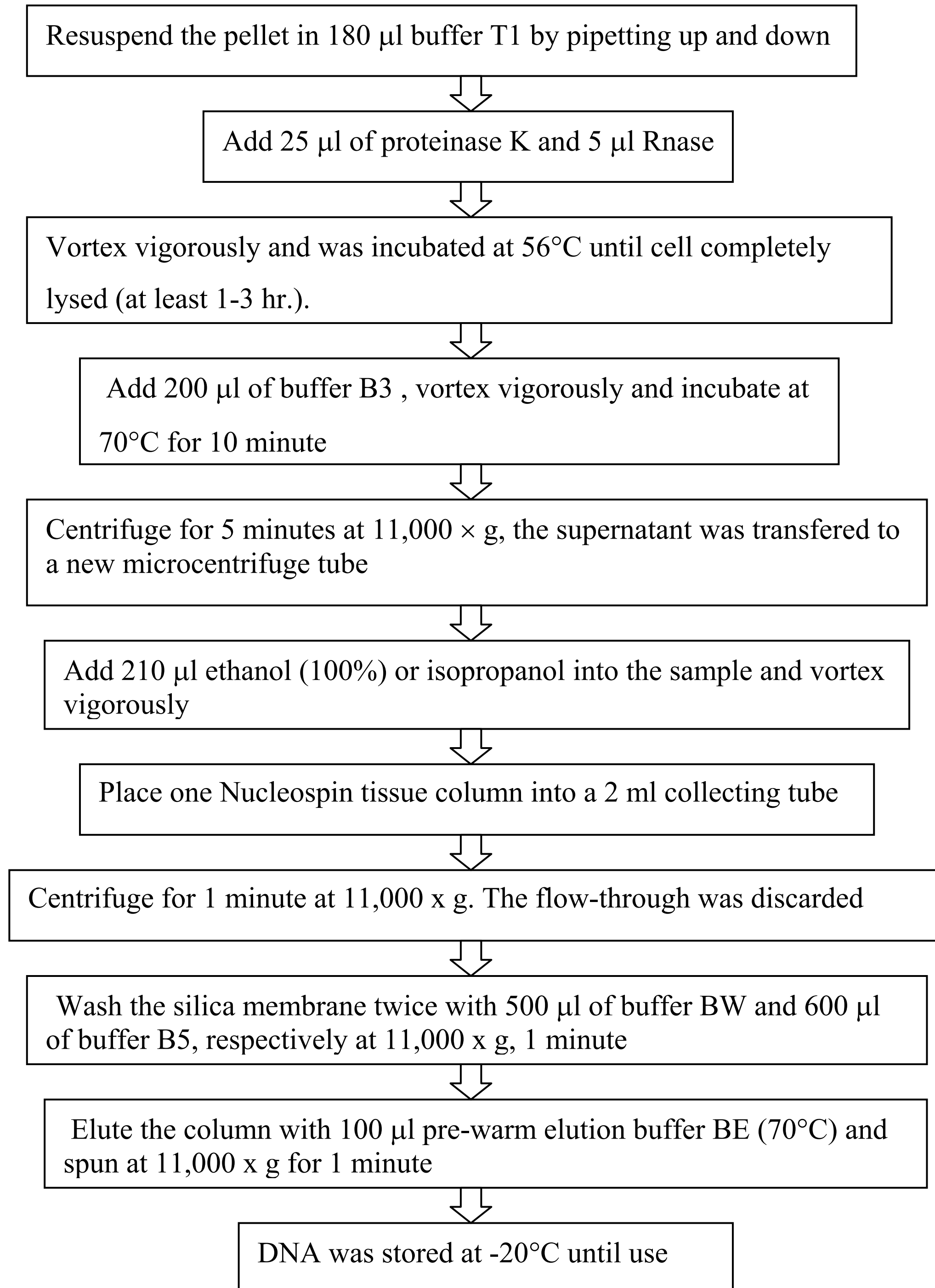


Figure 12 Methodology flow chart of DNA extraction

LAMP primers

A *Acanthamoeba* spp. specific LAMP primer set consisting of primers F3, B3,FIP, and BIP was designed using the Primer Explorer program to amplify the 18S rRNA from GenBank (accession no.AY694145.1 *Acanthamoeba* spp. 18S ribosomal RNA gene)

Teble 5 LAMP primers

Target gene	Region*	5′ pos	3′ pos	bp	Sequence
18S rRNA	F3	145	169	25	GGGGCATTAATATTTAATTGTCAGA
	B3	303	320	18	TCTCCTAATCGCTGGTCG
	FIP			47	GAAAACATCCTTGGCAGATGCT GGTGAAATTCTTGGATTTATGAAAG
	BIP			46	AATCAAGAACGAAAGTTAGGGGAT ATCGTTTATGGTTAAGACTACG
	F2	170	194	25	GGTGAAATTCTTGGATTTATGAAAG
	F1c	210	231	22	GAAAACATCCTTGGCAGATGCT
	B2	279	300	22	ATCGTTTATGGTTAAGACTACG
	B1c	235	258	24	AATCAAGAACGAAAGTTAGGGGAT

* FIP, forward inner primer; F3, forward outer primer; BIP, backward inner primer; B3, backward primer.

Source: http://www.loopamp.eiken.co.jp/e/lamp/anim.htm [Accessed Nov. 11, 2006]

AAAATTAGAGTGTTCAAAGCAGGCAGATCCAATTTTCTGCCACCGAATACATTAGCATGGGATAATA

ATAGGACCCTGTCCTCCTATTTTCAGTTGGTTTTGGCAGCGCGAGGACTAGGGTAATGATTAATAATA

F3 F2
GTTGGGGGCATTAATATTTAATTGTCAGA GGTGAAATTCTTGGATTTATGAAAGATTAACTTCTGCA

F1c B1c
AGCATCTGCCAAGGATGTTTTCATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATCAGATAC
C

B2 B3
GTCGTAGTCTTAACCATAAACGATGC CGACCAGCGATTAGGAGACGTTGAATACAAAACACCACCA

TCGGTGCGGTCGTCCTTGGCGGTTGGTCTTCAAAAGCCAGCGGCGGGGNCGGCTTAGCCCGGTGGCA

CCGGTGAA

Figure 13 Sequence of primers FIP, BIP, F3 and B3 which were used in LAMP assay. Locations of primer-binding regions in the reference sequence (GenBank accession no.AY694145.1 *Acanthamoeba* spp. 18S ribosomal RNA gene) are indicated by arrows. Only the positive sense of the reference sequence is shown.

Source: http://www.loopamp.eiken.co.jp/e/lamp/anim.htm [Accessed Nov. 11, 2006]

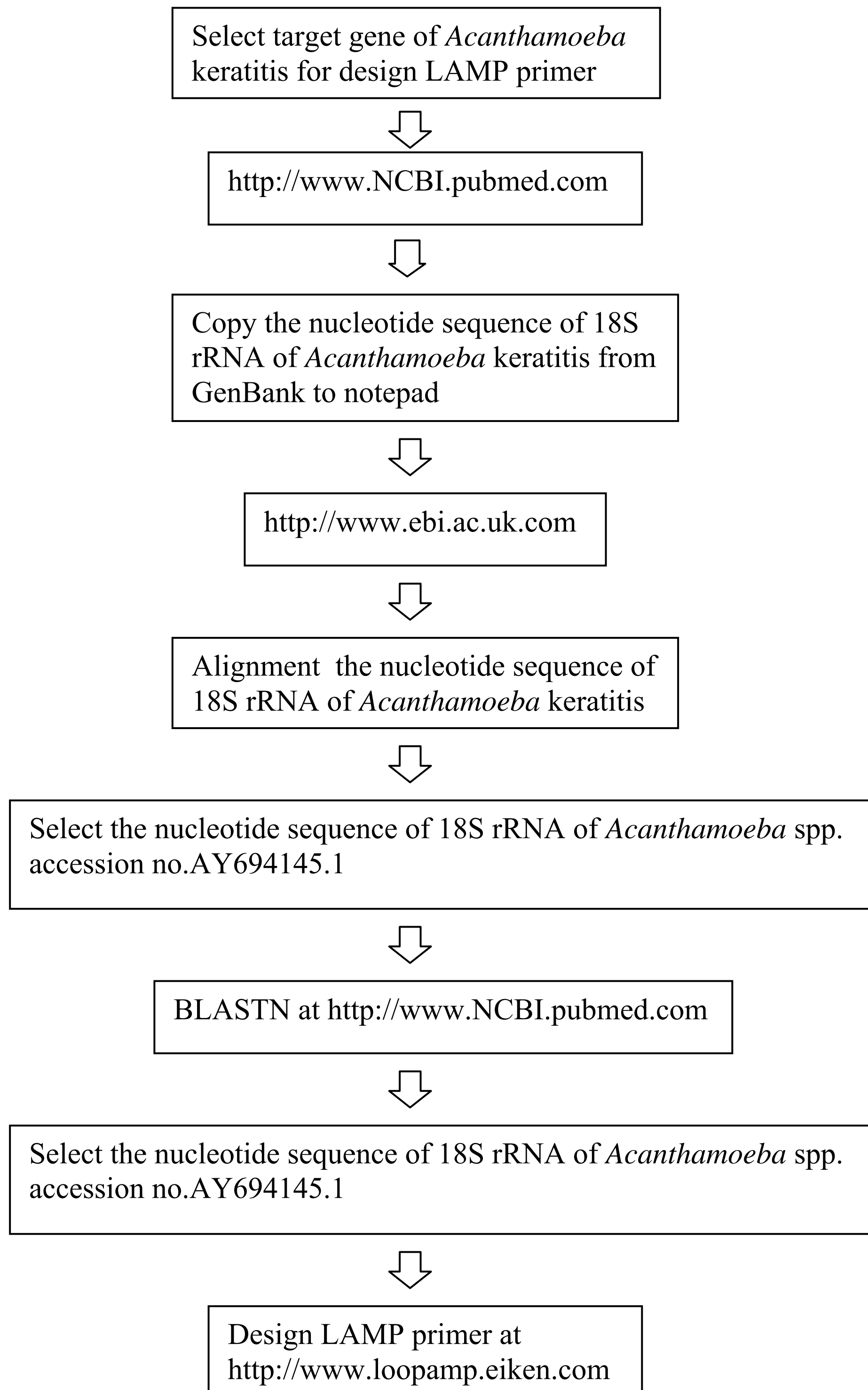


Figure 14 Methodology flow chart of LAMP primer design

RID: 1166197077-4209-146631871458.BLASTQ4
Database: All GenBank+EMBL+DDBJ+PDB sequences (but no EST, STS, GSS,environmental samples or phase 0, 1 or 2 HTGS sequences) 4,658,713 sequences; 18,685,068,004 total letters
Query = Length = 417

Distribution of 153 Blast Hits on the Query Sequence

Sequences producing significant alignments:	Score (Bits)	E Value
gi|33330339|gb|AF534144.1| Acanthamoeba sp. strain L595/97 18...	821	0.0
gi|56786208|gb|AY694145.1| Acanthamoeba sp. L1629/99 18S ribo...	821	0.0
gi|56786206|gb|AY694143.1| Acanthamoeba sp. L1629/99 18S ribo...	821	0.0
gi|56786201|gb|AY694138.1| Acanthamoeba sp. L565/97 18S ribos...	821	0.0
gi|507405|gb|U07409.1|ASU07409 Acanthamoeba sp. ATCC 50369 18...	793	0.0
gi|507397|gb|U07401.1|ACU07401 Acanthamoeba castellanii CDC:0184	781	0.0
gi|73623513|gb|DQ087314.1| Acanthamoeba sp. S22 18S ribosomal RN	773	0.0
gi|13027721|gb|AY026747.1| Acanthamoeba sp. isolate U/E3 smal...	765	0.0
gi|27883925|gb|AY148963.1| Acanthamoeba sp. KA/E17 18S riboso...	749	0.0
gi|73623518|gb|DQ087319.1| Acanthamoeba sp. S24 18S ribosomal RN	733	0.0
gi|73623517|gb|DQ087318.1| Acanthamoeba sp. 343TRE 18S riboso...	733	0.0
gi|73623516|gb|DQ087317.1| Acanthamoeba sp. S16 18S ribosomal RN	733	0.0
gi|73623515|gb|DQ087316.1| Acanthamoeba sp. S26 18S ribosomal RN	733	0.0
gi|73623514|gb|DQ087315.1| Acanthamoeba sp. S12 18S ribosomal RN	733	0.0
gi|33330351|gb|AF534156.1| Acanthamoeba sp. from Pakistan 18S...	733	0.0
gi|33330334|gb|AF534139.1| Acanthamoeba culbertsoni strain Di...	733	0.0
gi|33330330|gb|AF534135.1| Acanthamoeba castellanii strain AT...	733	0.0
gi|56118006|gb|AY661859.1| Acanthamoeba sp. strain KL 18S rib...	733	0.0
gi|29650248|gb|AY237735.1| Acanthamoeba polyphaga 18S ribosomal	733	0.0
gi|507406|gb|U07410.1|ASU07410 Acanthamoeba sp. ATCC 50497 18...	733	0.0
gi|10801184|gb|AF260720.1|AF260720 Acanthamoeba rhysodes 18S ...	733	0.0
gi|10801182|gb|AF260718.1|AF260718 Acanthamoeba lugdunensis 1...	733	0.0
gi|27085440|gb|AY173008.1| Acanthamoeba sp. KA/MSG16 18S ribo...	733	0.0
gi|56718203|gb|AY703018.1| Acanthamoeba sp. CDC # VE67/H7 sma...	733	0.0
gi|56718189|gb|AY703004.1| Acanthamoeba sp. CDC # V390 small ...	733	0.0
gi|56718188|gb|AY703003.1| Acanthamoeba sp. CDC # V388 small ...	733	0.0
gi|56718187|gb|AY703002.1| Acanthamoeba sp. CDC # V382 small ...	733	0.0
gi|56718184|gb|AY702999.1| Acanthamoeba sp. CDC # V328 small ...	733	0.0
gi|56718175|gb|AY702990.1| Acanthamoeba sp. CDC # V087 small ...	733	0.0

Figure 15 Homology analysis of *Acanthamoeba* spp. nucleotide sequence, obtained from BLAST search program at the National Center of Biotechnology Information (NCBI), U.S.A.

RID: 1166197077-4209-146631871458.BLASTQ4
Database: All GenBank+EMBL+DDBJ+PDB sequences (but no EST, STS, GSS,environmental samples or phase 0, 1 or 2 HTGS sequences) 4,658,713 sequences; 18,685,068,004 total letters
Query = Length = 417

Distribution of 153 Blast Hits on the Query Sequence

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gi|33330339|gb|AF534144.1| Acanthamoeba sp. strain L595/97 18...	821	0.0
gi|56786208|gb|AY694145.1| Acanthamoeba sp. L1629/99 18S ribo...	821	0.0
gi|56786206|gb|AY694143.1| Acanthamoeba sp. L1629/99 18S ribo...	821	0.0
gi|56786201|gb|AY694138.1| Acanthamoeba sp. L565/97 18S ribos...	821	0.0
gi|507405|gb|U07409.1|ASU07409 Acanthamoeba sp. ATCC 50369 18...	793	0.0
gi|507397|gb|U07401.1|ACU07401 Acanthamoeba castellanii CDC:0184	781	0.0
gi|73623513|gb|DQ087314.1| Acanthamoeba sp. S22 18S ribosomal RN	773	0.0
gi|13027721|gb|AY026747.1| Acanthamoeba sp. isolate U/E3 smal...	765	0.0
gi|27883925|gb|AY148963.1| Acanthamoeba sp. KA/E17 18S riboso...	749	0.0
gi|73623518|gb|DQ087319.1| Acanthamoeba sp. S24 18S ribosomal RN	733	0.0
gi|73623517|gb|DQ087318.1| Acanthamoeba sp. 343TRE 18S riboso...	733	0.0
gi|73623516|gb|DQ087317.1| Acanthamoeba sp. S16 18S ribosomal RN	733	0.0
gi|73623515|gb|DQ087316.1| Acanthamoeba sp. S26 18S ribosomal RN	733	0.0
gi|73623514|gb|DQ087315.1| Acanthamoeba sp. S12 18S ribosomal RN	733	0.0
gi|33330351|gb|AF534156.1| Acanthamoeba sp. from Pakistan 18S...	733	0.0
gi|33330334|gb|AF534139.1| Acanthamoeba culbertsoni strain Di...	733	0.0
gi|33330330|gb|AF534135.1| Acanthamoeba castellanii strain AT...	733	0.0
gi|56118006|gb|AY661859.1| Acanthamoeba sp. strain KL 18S rib...	733	0.0
gi|29650248|gb|AY237735.1| Acanthamoeba polyphaga 18S ribosomal	733	0.0
gi|507406|gb|U07410.1|ASU07410 Acanthamoeba sp. ATCC 50497 18...	733	0.0
gi|10801184|gb|AF260720.1|AF260720 Acanthamoeba rhysodes 18S ...	733	0.0
gi|10801182|gb|AF260718.1|AF260718 Acanthamoeba lugdunensis 1...	733	0.0
gi|27085440|gb|AY173008.1| Acanthamoeba sp. KA/MSG16 18S ribo...	733	0.0
gi|56718203|gb|AY703018.1| Acanthamoeba sp. CDC # VE67/H7 sma...	733	0.0
gi|56718189|gb|AY703004.1| Acanthamoeba sp. CDC # V390 small ...	733	0.0
gi|56718188|gb|AY703003.1| Acanthamoeba sp. CDC # V388 small ...	733	0.0
gi|56718187|gb|AY703002.1| Acanthamoeba sp. CDC # V382 small ...	733	0.0
gi|56718184|gb|AY702999.1| Acanthamoeba sp. CDC # V328 small ...	733	0.0
gi|56718175|gb|AY702990.1| Acanthamoeba sp. CDC # V087 small ...	733	0.0

Figure 15 Homology analysis of *Acanthamoeba* spp. nucleotide sequence, obtained from BLAST search program at the National Center of Biotechnology Information (NCBI), U.S.A. (cont.)

RID: 1166197077-4209-146631871458.BLASTQ4
Database: All GenBank+EMBL+DDBJ+PDB sequences (but no EST, STS, GSS,environmental samples or phase 0, 1 or 2 HTGS sequences) 4,658,713 sequences; 18,685,068,004 total letters
Query = Length = 417

Distribution of 153 Blast Hits on the Query Sequence

Sequences producing significant alignments:	Score (Bits)	E Value
gi|27883916|gb|AY148954.1| Acanthamoeba sp. KA/E5 18S ribosom...	720	0.0
gi|507411|gb|U07415.1|APU07415 Acanthamoeba polyphaga JAC/S2 AT	720	0.0
gi|516524|gb|U07402.1|APU07402 Acanthamoeba polyphaga CDC:0884:	720	0.0
gi|507410|gb|U07414.1|ACU07414 Acanthamoeba castellanii Ma ATCC	720	0.0
gi|73623503|gb|DQ087304.1| Acanthamoeba sp. S9 18S ribosomal RNA	712	0.0
gi|73623500|gb|DQ087301.1| Acanthamoeba sp. S31 18S ribosomal RN	712	0.0
gi|73623499|gb|DQ087300.1| Acanthamoeba sp. S15 18S ribosomal RN	712	0.0
gi|73623498|gb|DQ087299.1| Acanthamoeba sp. S21 18S ribosomal RN	712	0.0
gi|73623497|gb|DQ087298.1| Acanthamoeba sp. 413GAB 18S riboso...	712	0.0
gi|73623496|gb|DQ087297.1| Acanthamoeba sp. 222BAL 18S riboso...	712	0.0
gi|73623495|gb|DQ087296.1| Acanthamoeba sp. S6 18S ribosomal RNA	712	0.0
gi|45386041|gb|AY549559.1| Acanthamoeba sp. AC309 18S ribosomal	712	0.0
gi|45386040|gb|AY549558.1| Acanthamoeba sp. ATCC 30868 (AC021...	712	0.0
gi|45386039|gb|AY549557.1| Acanthamoeba sp. ATCC 30871 (AC010...	712	0.0
gi|33330332|gb|AF534137.1| Acanthamoeba castellanii strain AT...	712	0.0
gi|13162668|gb|AY026251.1| Acanthamoeba sp. isolate U/E8R sma...	712	0.0
gi|13162667|gb|AY026250.1| Acanthamoeba sp. isolate U/E8X sma...	712	0.0
gi|10801188|gb|AF260724.1|AF260724 Acanthamoeba castellanii s...	712	0.0
gi|10801186|gb|AF260722.1|AF260722 Acanthamoeba hatchetti str...	712	0.0
gi|10801183|gb|AF260719.1|AF260719 Acanthamoeba palestinensis...	712	0.0
gi|13560696|gb|AF349044.1| Acanthamoeba sp. KA/E6 18S ribosom...	712	0.0
gi|27085439|gb|AY173007.1| Acanthamoeba sp. KA/MSG15 18S ribo...	712	0.0
gi|27085436|gb|AY173004.1| Acanthamoeba sp. KA/MSG4 18S ribos...	712	0.0
gi|13027723|gb|AY026749.1| Acanthamoeba sp. isolate U/E10 sma...	712	0.0
gi|2979655|gb|AF019055.1| Acanthamoeba sp. Liu-E1 18S ribosom...	712	0.0
gi|27883924|gb|AY148962.1| Acanthamoeba sp. KA/E16 18S riboso...	712	0.0
gi|27883923|gb|AY148961.1| Acanthamoeba sp. KA/E15 18S riboso...	712	0.0
gi|27883922|gb|AY148960.1| Acanthamoeba sp. KA/E14 18S riboso...	712	0.0
gi|27883919|gb|AY148957.1| Acanthamoeba sp. KA/E10 18S riboso...	712	0.0
gi|27883918|gb|AY148956.1| Acanthamoeba sp. KA/E8 18S ribosom...	712	0.0
gi|27883917|gb|AY148955.1| Acanthamoeba sp. KA/E7 18S ribosom...	712	0.0
gi|2979659|gb|AF019059.1| Acanthamoeba sp. Rodriguez 18S ribo...	712	0.0
gi|7672554|gb|AF140714.1| Acanthamoeba sp. strain 4337 clone ...	712	0.0
gi|5114144|gb|AF114438.1|AF114438 Acanthamoeba castellanii 18...	712	0.0
gi|2979660|gb|AF019060.1|AF019060 Acanthamoeba hatchetti 2AX1...	712	0.0
gi|2979661|gb|AF019061.1|AF019061 Acanthamoeba polyphaga Page...	712	0.0

Figure 15 Homology analysis of *Acanthamoeba* spp. nucleotide sequence, obtained from BLAST search program at the National Center of Biotechnology Information (NCBI), U.S.A.(cont.)

gi|56786208|gb|AY694145.1| Acanthamoeba sp. L1629/99 18S ribosomal RNA gene, complete sequence
Length = 417
Score = 821 bits (414), Expect = 0.0
Identities = 417/417 (100%),
Gaps = 0/417 (0%) Strand=Plus/Plus

```
Query     1  AAAATTAGAGTGTTCAAAGCAGGCAGATCCAATTTTCTGCCACCGAATACATTAGCATGG   60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject   1  AAAATTAGAGTGTTCAAAGCAGGCAGATCCAATTTTCTGCCACCGAATACATTAGCATGG   60

Query    61  GATAATGGAATAGGACCCTGTCCTCCTATTTTCAGTTGGTTTTGGCAGCGCGAGGACTAG  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject  61  GATAATGGAATAGGACCCTGTCCTCCTATTTTCAGTTGGTTTTGGCAGCGCGAGGACTAG  120

Query   121  GGTAATGATTAATAGGGATAGTTGGGGGCATTAATATTTAATTGTCAGAGGTGAAATTCT  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject 121  GGTAATGATTAATAGGGATAGTTGGGGGCATTAATATTTAATTGTCAGAGGTGAAATTCT  180

Query   181  TGGATTTATGAAAGATTAACTTCTGCGAAAGCATCTGCCAAGGATGTTTTCATTAATCAA  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject 181  TGGATTTATGAAAGATTAACTTCTGCGAAAGCATCTGCCAAGGATGTTTTCATTAATCAA  240

Query   241  GAACGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAACCATAAACGAT  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject241  GAACGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAACCATAAACGAT  300

Query   301  GCCGACCAGCGATTAGGAGACGTTGAATACAAAACACCACCATCGGTGCGGTCGTCCTTG  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject 301  GCCGACCAGCGATTAGGAGACGTTGAATACAAAACACCACCATCGGTGCGGTCGTCCTTG  360

Query   361  GCGGTTGGTCTTCAAAAGCCAGCGGCGGGGNCGGCTTAGCCCGGTGGCACCGGTGAA  417
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject 361  GCGGTTGGTCTTCAAAAGCCAGCGGCGGGGNCGGCTTAGCCCGGTGGCACCGGTGAA  417
```

Figure 16 Length of 417 of *Acanthamoeba* spp. nucleotide (AY694145.1) 18S ribosomal RNA gene, complete sequence of genomic with 100% (417/417) from BLAST search program at the National Center of Biotechnology Information (NCBI), U.S.A.

LAMP reaction

1. LAMP was performed in 25 µl of a mixture containing 2 µl of the extracted DNA, 40 pmol each of FIP and BIP, 5 pmol each of F3 and B3, 1.4 mM deoxynucleoside triphosphates, 0.8 M betaine and 1 µl of *Bst* DNA polymerase in 1x buffer [20 mM Tris-HCL (pH 8.8), 10 mM KCL, 10 mM $(NH4)_2SO_4$, 8 mM $MgSO_4$, and 0.1% Tween 20].
2. The LAMP reaction was performed for 60 min at 65°C. Inactivation for 2 min at 80°C was performed after the last cycle.
3. After incubation, turbidities of the LAMP reactions were inspected visually. The LAMP products were confirmed analysis by Fluorescence dye and electrophoresis with 1.5% agarose gels, followed by ethidium bromide staining and photography.

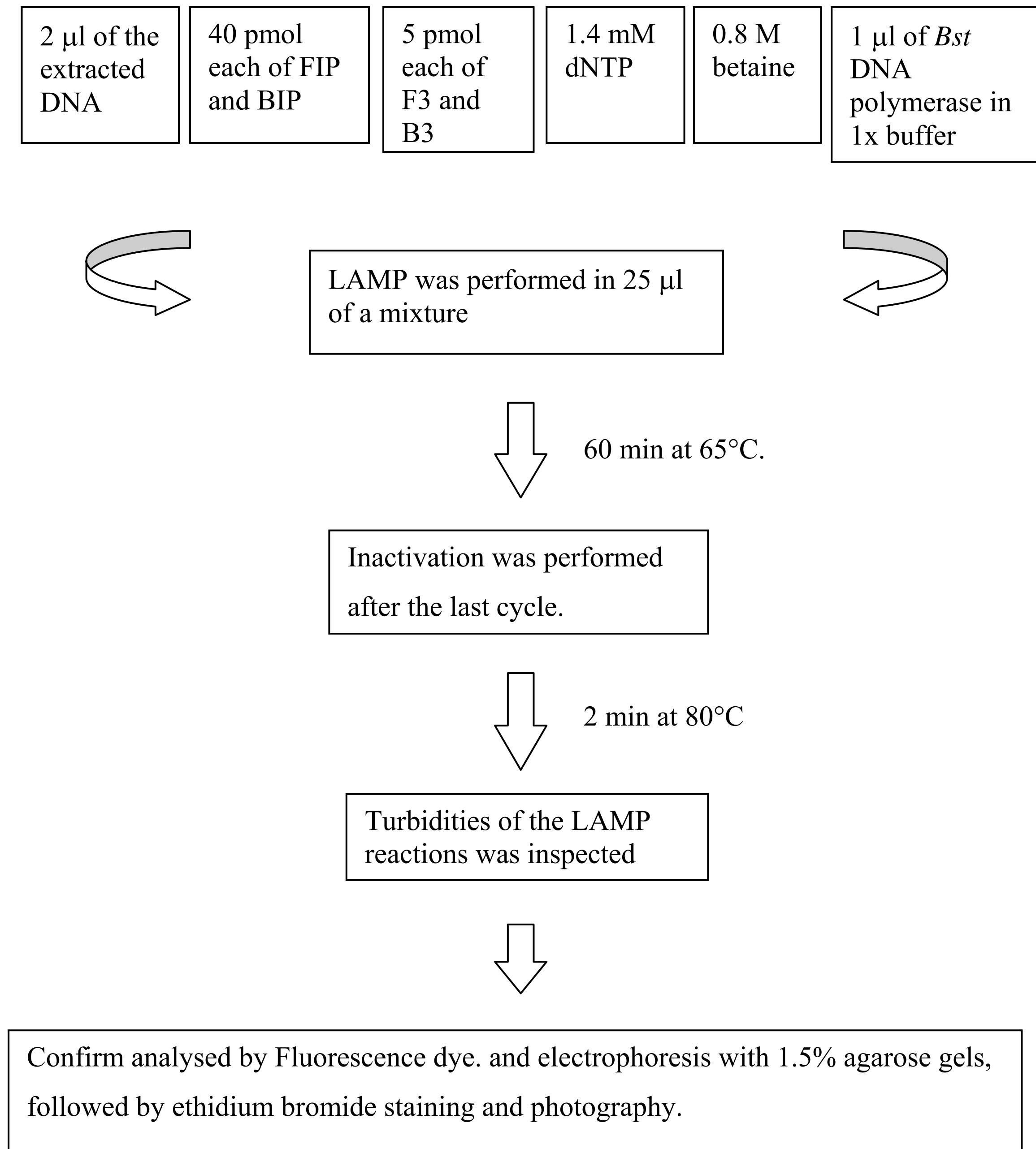


Figure 17 Methodology flow chart of LAMP reaction

Comparison of low limited detection in LAMP and Real-time PCR Assay.

Since the outer primer pair, designated F3 and B3, can also be used for Real-time PCR, the same target gene was amplified from serially diluted total *Acanthamoeba* spp. DNA by both LAMP and Real-time PCR Assay and the low limited detection of two methods were compared.

Total DNA from *Acanthamoeba* spp. were serially diluted from 10 ng to 0.001 pg and amplified by LAMP and Real-time PCR.

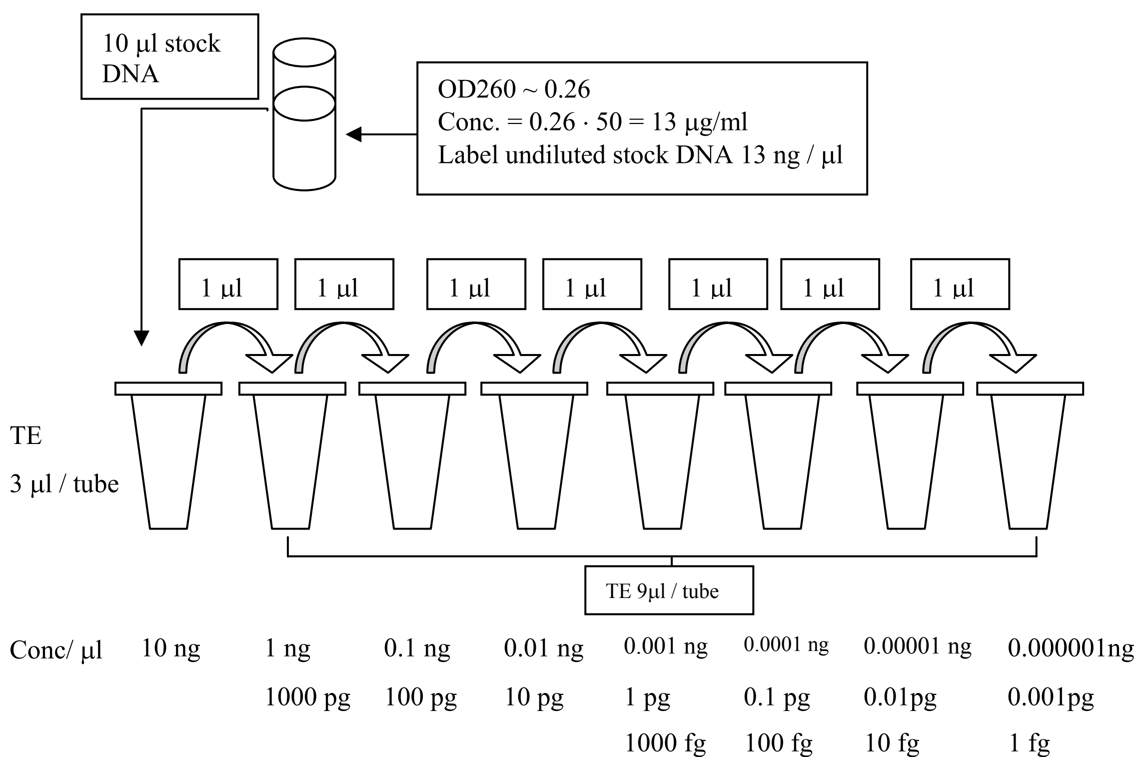


Figure 18 Serial dilution of *Acanthamoeba* spp. DNA.

Real-time PCR reaction

Real-time PCR was performed and analyzed using the 2xQuantiTect SYBR Green sequence detector under the following conditions.

Primers were designed to amplify *Acanthamoeba* spp. specific region of the 18S rRNA gene.

Teble 6 Real-time PCR primers for the detection of *Acanthamoeba* spp.

Target gene	Region*	5′ pos	3′ pos	bp	Sequence
18S rRNA	F3	145	169	25	GGGGCATTAATATTTAATTGTCAGA
	B3	303	320	18	TCTCCTAATCGCTGGTCG

* F3, forward outer primer; B3, backward primer.

Source: http://www.loopamp.eiken.co.jp/e/lamp/anim.htm [Accessed Nov. 11, 2006]

In this study we used the specific primers for the detection of *Plasmodium falciparum, Plasmodium vivax* and *Entamoeba histolytica* in order to show that they had different melting curves from that of *Acanthamoeba* spp..

Teble 7 Real-time PCR primers for the detection of *Plasmodium falciparum* and *Plasmodium vivax*

Target gene	bp	Sequence
18S rRNA	18	5'-TAA CGA ACG AGA TCT TAA-3'
	18	5'-GTT CCT CTA AGA AGC TTT-3'

Teble 8 Real-time PCR primers for detection of *Enthamoeba histolytica*

Target gene	bp	Sequence
18S rRNA	29	5'-GTG CTG AAA CCT AGC TAT TGT AAC TCA GT -3'
	25	5'-CAC TAG AAA AAG CAA ACC TGA AAG G -3'

Teble 9 Master Mix for Real-time PCR

Reagents	Volume (µl)
RNase free water	3.80
$MgCl_2$	4.00
2x QuantiTect SYBR Green PCR master Mix	10.00
Primer - F3	0.10
- B3	0.10
Template	2.00
Final volume	20.00

Real-time PCR reaction

1. Thaw 2x QuantiTect SYBR PCR Master Mix (kept frozen at -20°C), template DNA, primers, and RNase-free water. Mix the individual solutions.
2. Prepare a master mix according to Table 9.
3. Mix the master mix thoroughly and appropriate volumes into PCR capillaries.
4. Add template DNA (≤1µg/reaction) to the individual PCR capillaries.
5. Program the Light Cycle and set fluorescence gains

Data acquisition was performed during the extension step. After performing a melting curve analysis, an additional data acquisition step for further runs with the same target was integrated.

6. Place the PCR capillaries in LightCycler machine and start the cycling program.
7. Perform a melting curve analysis of PCR product.

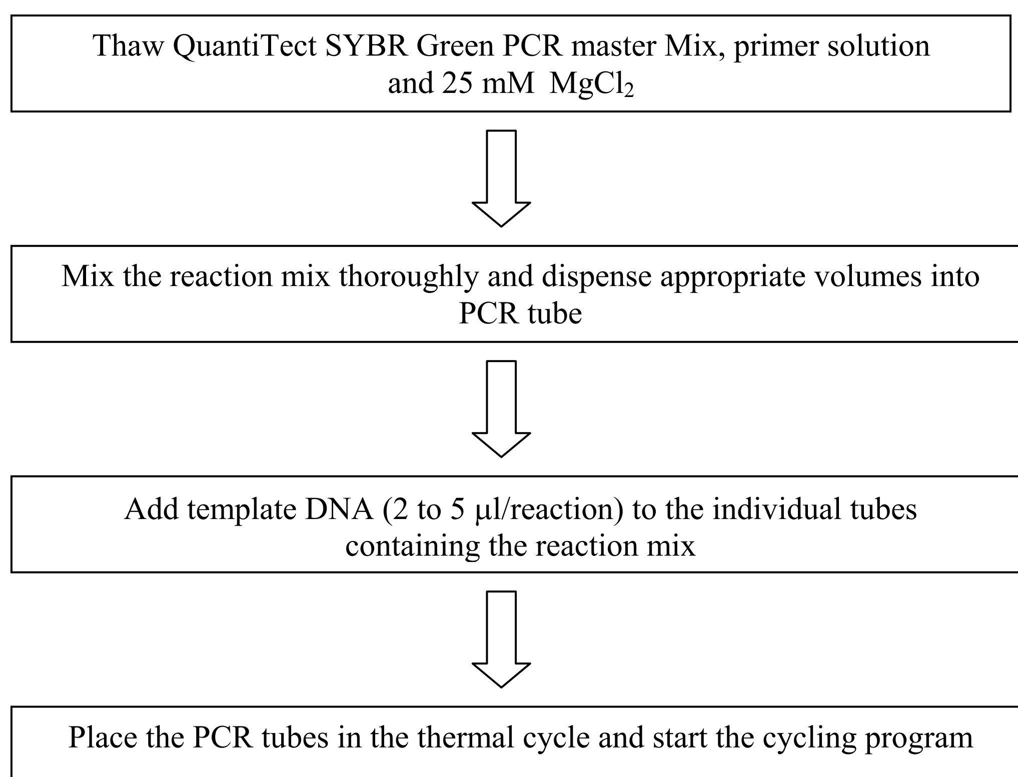
We highly recommend routinely performing this analysis step in order to verify specificity and identity of the PCR products. Melting curve analysis is an analysis step built into the software of the LightCycler. Follow instructions provided by the supplier. Generally, melting curve data between 55°C and 95°C was acquired.

The T_m of PCR product depends on buffer composition and salt concentration. Values obtained using other reagents, depending on primer design and copy number of target, primer-dimer occurrence. These were distinguished from the specific product through their lower melting points.

8. Optional: Repeat the previous run, including an additional data acquisition step.

In order to suppress fluorescence readings caused by generation of primer-dimers, and additional data acquisition step can be added to the 3-step cycling protocol. The temperature should be above the T_m of primer-dimers but approximately 3°C below the T_m of the specific PCR product. This method can increase the dynamic rang and reliability of quantification by several orders of magnitude if primer-dimers are co-amplified.

9. Optional: Check the specificity of PCR products using agarose gel electrophoresis.



The PCR conditions were as follows:

Step 1	95°C for 15 min	Initial denaturation
Step 2	94°C for 15 sec	Annealing
Step 3	56°C for 30 sec	Extention
Step 4	72°C for 30 sec	Denaturation
Step 5	Repeat the steps 2-4 a total of 40 times	

The level of fluorescence will be captured at the end of each extension step. Immediately after amplification a melt program consisting of 2 min at 95°C, 2 min at 55°C, and a stepwise temperature increase of 0.2°C/s until 90°C, with fluorescence acquisition at each temperature transition is performed for speciation.

The T_m values: 57.32 to 57.44°C

Data analysis

Statistical analysis.

1. Validity of test

The LAMP and culture results from 150 samples will be used for determination of sensitivity and specificity of LAMP results, in this study using culture results as the "gold standard."

		LAMP	
Culture		Positive	Negative
	Positive	True positive	False positive
	Negative	False negative	True negative

Sensitivity = (True positive/ True positive + False negative) × 100

Specificity = (True negative/ True negative + False positive) × 100

1. Prevalence Rate

$$\text{Prevalence Rate} = \frac{\text{number of } \textit{Acanthamoeba} \text{ spp. positive}}{\text{Total number of samples}} \times 100$$

CHAPTER IV
RESULTS

The results consisted of 4 parts:

Part 1: Observation of the proper temperature for *Acanthamoeba* spp. growth in contact lens storage cases.

Part 2: The results of *Acanthamoeba* spp. cultivation on non-nutrient agar, the comparison of sensitivity and specificity of LAMP method and culture technique for the detection of *Acanthamoeba* spp. from contact lens storage cases.

Part 3:The results of *Acanthamoeba* spp. detection by Loop-mediated isothermal amplification of DNA (LAMP).

Part 4: Comparison of low limited detection in LAMP and Real-time PCR Assay.

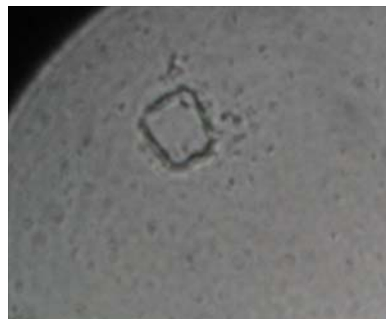
Collection of contact lens storage cases was conducted at Silapakorn University, Nakhornpathom Province. Laboratory works were performed at the Faculty of Public Health, Department of Parasitology, Mahidol University, Bangkok. This study was performed since March till October 2007.

Part 1 Observation of the proper temperature growth for *Acanthamoeba* spp. in contact lens storage cases.

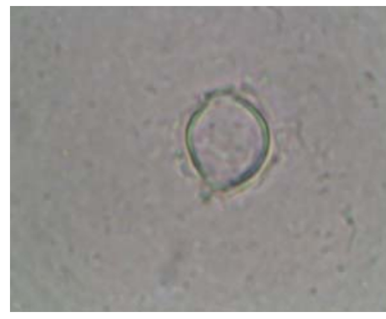
The cysts of *Acanthamoeba* spp. were dropped onto the contact lens storage cases containing contact lens solution and *E.coli*, incubated at various temperatures ranging from 36.8-37.2°C and observed for the presence of cysts and/or trophozoites for 7 days. The results are shown in table 7. The isolated trophozoites were subjected to DNA extraction procedure.

Table 10 The results of artificial infection of *Acanthamoeba* spp. to contact lens storage cases

Day	Date	Temperature	Results
2	15/6/07	36.8°C	Cysts of various *Acanthamoeba* spp. growing in contact lens storage cases.(Figure 19)
5	18/6/07	37°C	Cysts of various *Acanthamoeba* spp. growing in contact lens storage cases. (Figure 20)
7	20/6/07	37.1°C	Cysts of *Acanthamoeba* spp. showing stage of mitosis and nuclei. (Figure 21)
8	21/6/07	37.2°C	The *Acanthamoeba* trophozoites showing nuclei and contractile vacuole. (Figure 22)
9	22/6/07	37.1°C	The *Acanthamoeba* trophozoites showing contractile vacuole and acanthopodia. (Figure 23)
12	25/6/07	37°C	The *Acanthamoeba* trophozoites moving freely in water and presence of lobopodium and needle like fine projections of pseudopodia called acanthopodia.The average size of *Acanthamoeba* trophozoites was 25 µm. (Figure 24)



Cyst of group II (polyphagids)



Cyst of group III (culbertsonids)

Figure 19 Cysts of various *Acanthamoeba* spp. growing in contact lens storage cases.

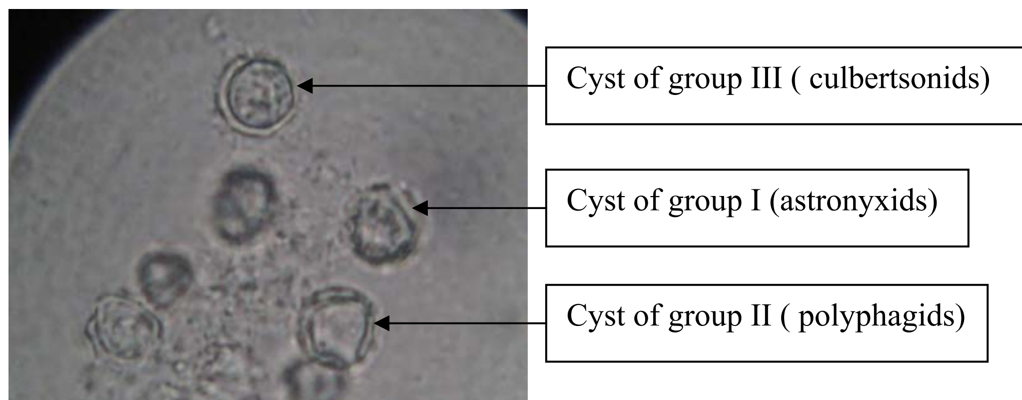


Figure 20 Cysts of various *Acanthamoeba* spp. growing in contact lens storage cases.

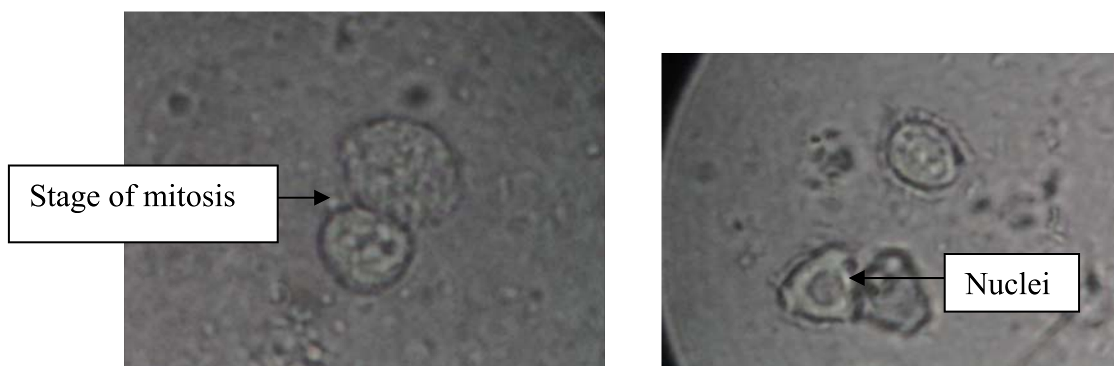


Figure 21 Cysts of *Acanthamoeba* spp. showing stage of mitosis and nuclei

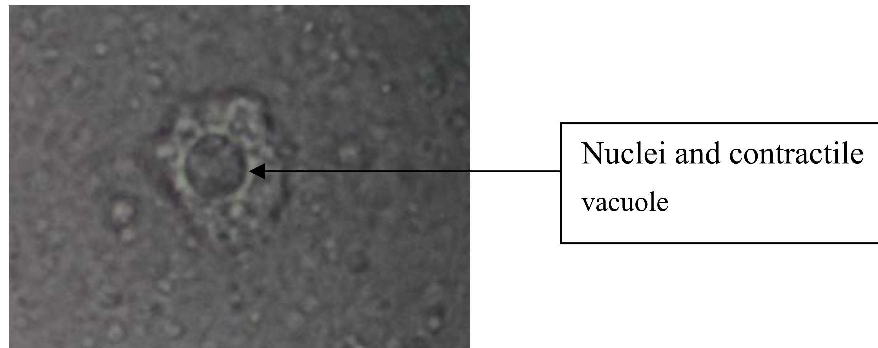


Figure 22 The *Acanthamoeba* trophozoites showing nuclei and contractile vacuole.

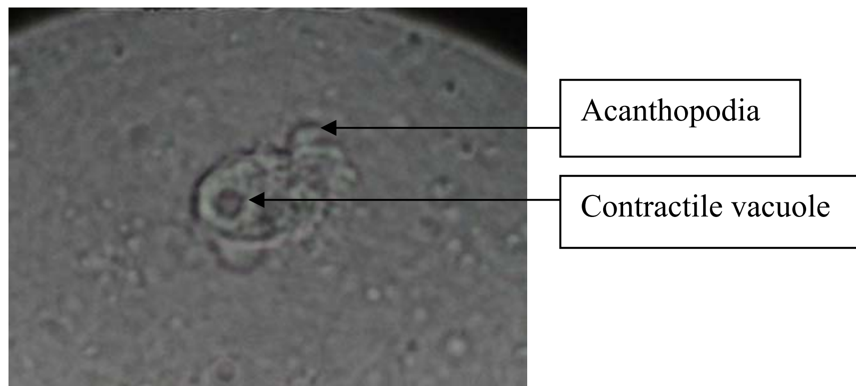


Figure 23 The *Acanthamoeba* trophozoites showing contractile vacuole and acanthopodia.

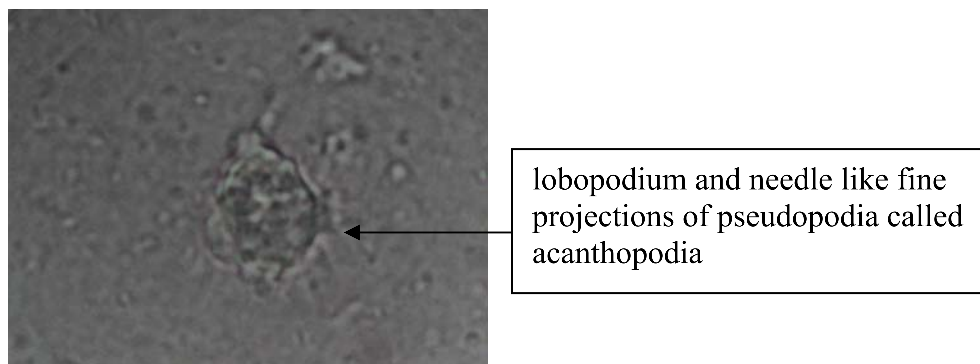


Figure 24 The *Acanthamoeba* trophozoites moving freely in water and presence of lobopodium and needle like fine projections of pseudopodia called acanthopodia.

Part 2 The results of *Acanthamoeba* spp. cultivation on non-nutrient agar, the comparison of sensitivity and specificity of LAMP method and culture technique for the detection of *Acanthamoeba* spp. from contact lens storage cases.

The prevalence of *Acanthamoeba* spp. in contact lens wearers in students at Silapakorn University.

The study was conducted at Silapakorn University, Nakhornpathom Province. Laboratoties were performed at the Faculty of Public Health, Department of Parasitology, Mahidol University, Bangkok.

All contact lens storage cases samples were collected in March till September 2007. 150 samples were obtained from students at Silapakorn University. *Acanthamoeba* spp. was found in 10 samples, the prevalence was 6.7 % (10/150). Cysts of the isolated amoebae were studied morphologically and devided into 3 different subgenera according to the characteristics previously described by Tsuruhara. The cysts were stored in amoeba saline at 4°C until used for further investigations.

Morphological classification

Trophozoites

The typical morphology of *Acanthamoeba* trophozoites moving freely in water is recognized by the presence of lobopodium and needle-like fine projections of pseudopodia called acanthopodia as shown in Figure 25. The average size of *Acanthamoeba* trophozoites was 25 µm, with the rang of 12-45 µm.

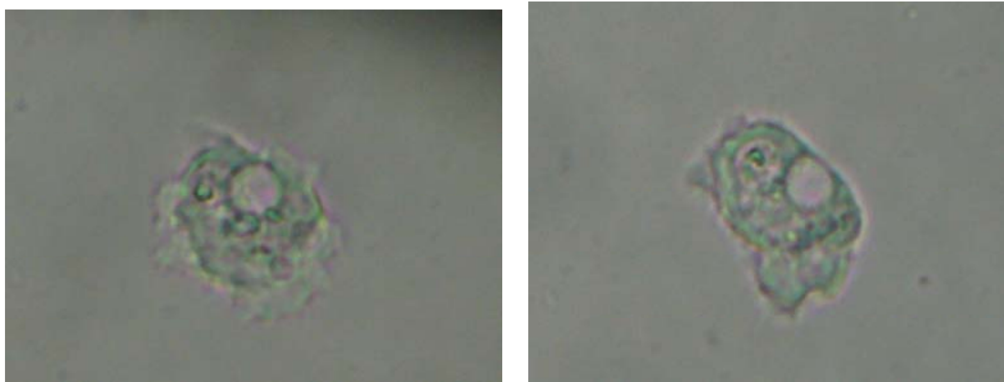


Figure 25 Trophozoites of *Acanthamoeba* spp.growing on non-nutrient agar**.**

Cysts

Based on the characteristics described by Tsuruhara , these *Acanthamoeba* isolates were found consisted of all three groups, i.e., Astronyxids (group I), Polyphagids (group II) and Culbertsonids (group III) as shown in Figure 26.

In summary, the average diameter of cysts in the group I is relatively large. They are ≥18 μm, while those of group II and group III are ≤ 18 μm.

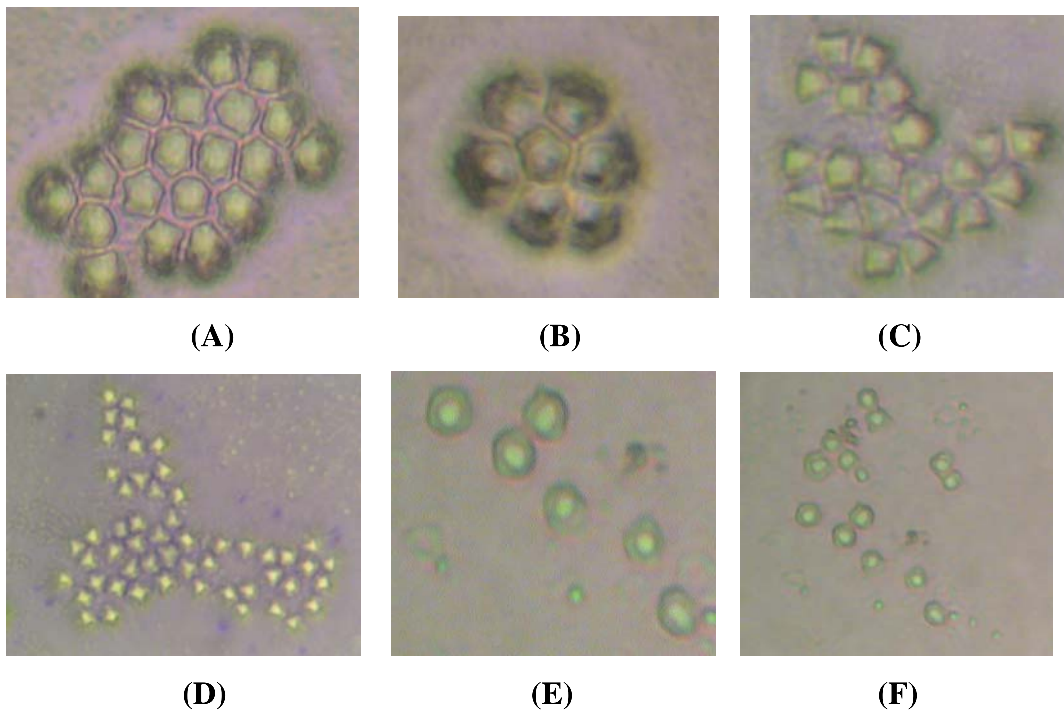


Figure 26 Cysts of various *Acanthamoeba* spp.growing on non-nutrient agar**.**

(A), (B): Astronyxids (group I)

(C), (D): Polyphagids (group II)

(E), (F): Culbertsonids (group III)

Part 3 The results of *Acanthamoeba* spp. detection by Loop-mediated isothermal amplification of DNA (LAMP) method.

Genomic DNA extraction

Acanthamoeba cysts were resistant to the ordinary cell lysis buffer in the DNA extraction process which could be observed under microscopic examination. In this study, we try to prepare *Acanthamoeba* cysts for DNA extraction by repeated freezing at -20°C for 1 hr and thawing at room temperature and observed the lysis of their cell walls under microscopic examination.

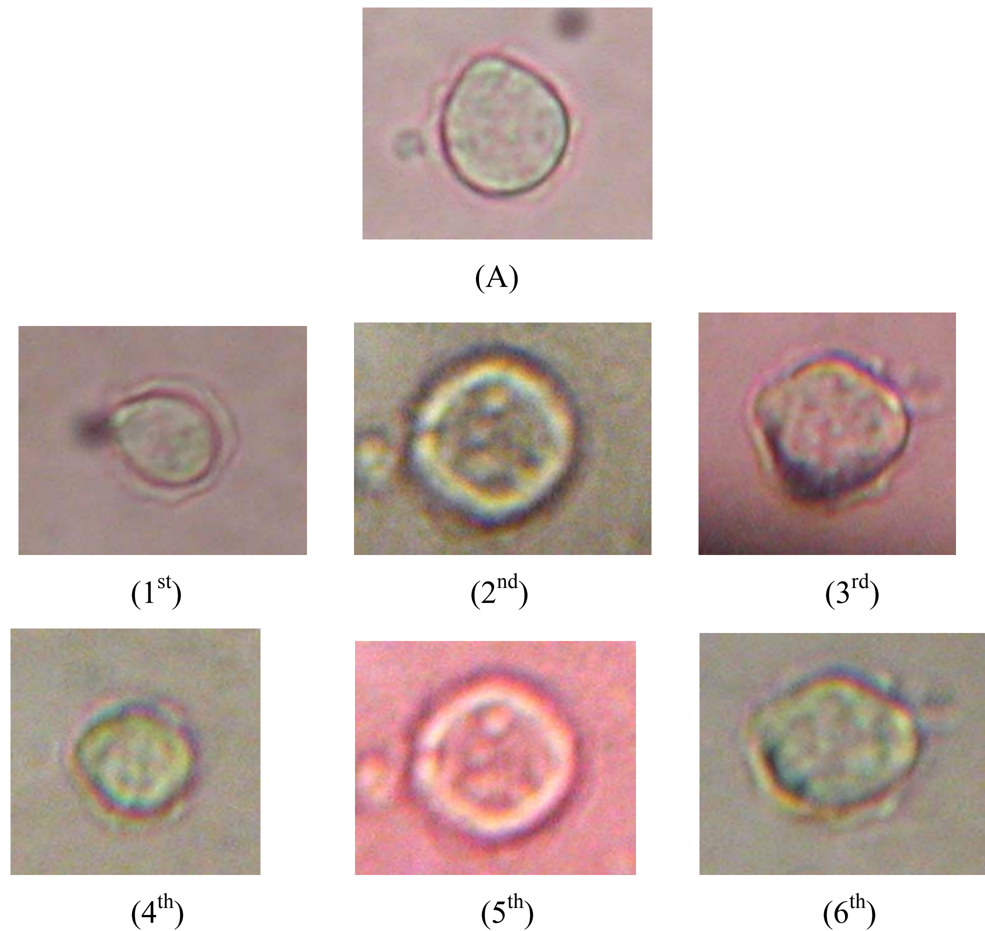


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Figure 27 Cysts of *Acanthamoeba* spp. before and after preparation for DNA extraction

(A): Cysts of *Acanthamoeba* spp. before preparation for DNA extraction

(1st): 1st preparation for DNA extraction

(2nd): 2nd preparation for DNA extraction

(3rd): 3rd preparation for DNA extraction

(4th): 4th preparation for DNA extraction

(5th): 5th preparation for DNA extraction

(6th): 6th preparation for DNA extraction

DNA was extracted from biofilm in contact lens storage cases with Neucleospin kit. The amount of DNA was measured using a spectrophotometer.

The results of *Acanthamoeba* spp. detection by LAMP from contact lens storage cases.

The LAMP method was examined using DNA extracted from biofilm in contact lens storage cases. Four sets of primers were designed for the 18S rRNA gene of *Acanthamoeba* spp. amplifications. To examine whether these sets of primers were able to amplify their target genes, LAMP reactions were conducted and analyzed from a white precipitate products and confirmation was analyze by fluorescence dye and electrophoreses in 1.5 % TBE agarose gel (Figure 28).

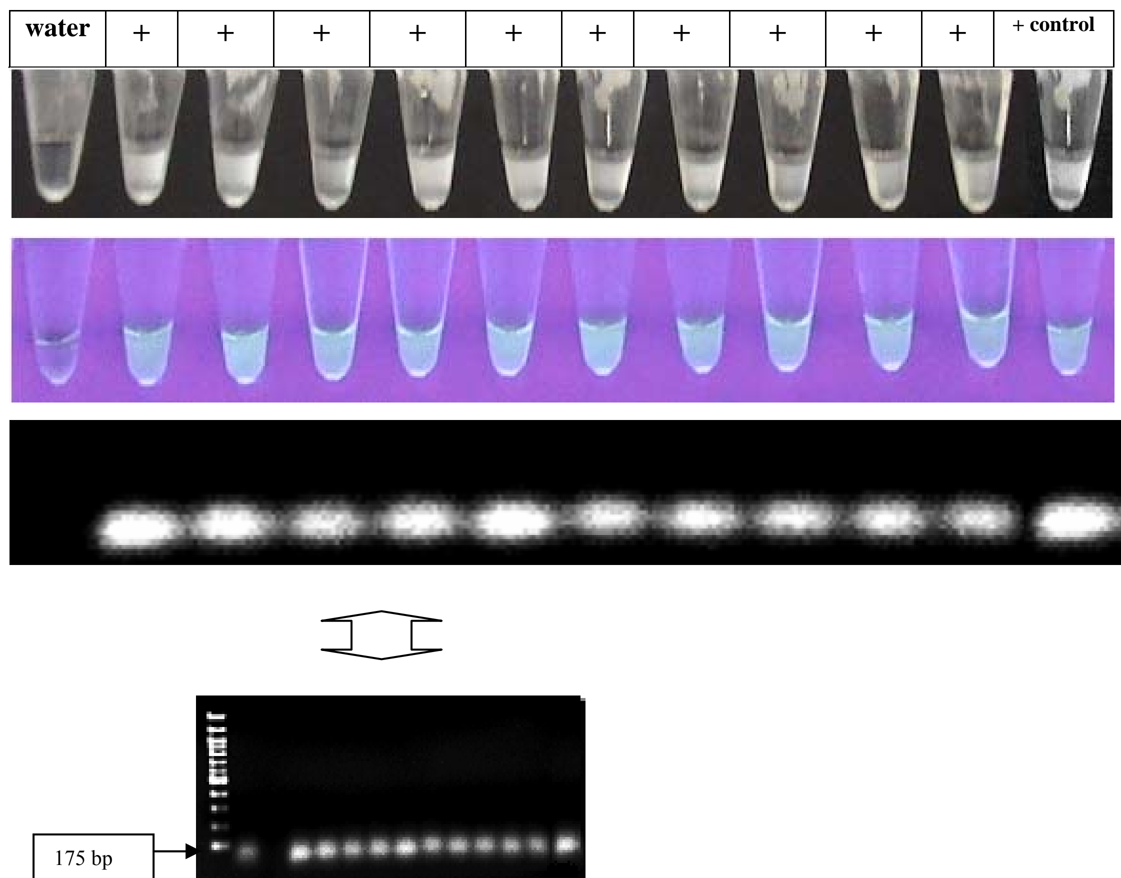

Figure 28 Detection of *Acanthamoeba* spp. in positive samples by LAMP assays. After incubation, turbidities of the LAMP reactions were inspected visually. Then confirmed analyzed by Fluorescene dye and electrophoreses in 1.5 % TBE agarose gel. The sizes of the 100 bp size markers are indicated on the left.

Part 4 Comparison of low limited detection in LAMP and Real-time PCR Assay.

Total DNAs from *Acanthamoeba* spp. were serially diluted from 10 ng to 0.001 pg and amplified by LAMP and real-time PCR. F3, B3, FIP and BIP are primer sets used in the LAMP reactions. The F3 and B3 primers in each LAMP primer set were used in the real-time PCR. The melting curve had shown the different curve between *Plasmodium falciparum, plasmodium vivax* and *Entamoeba histolytica*.

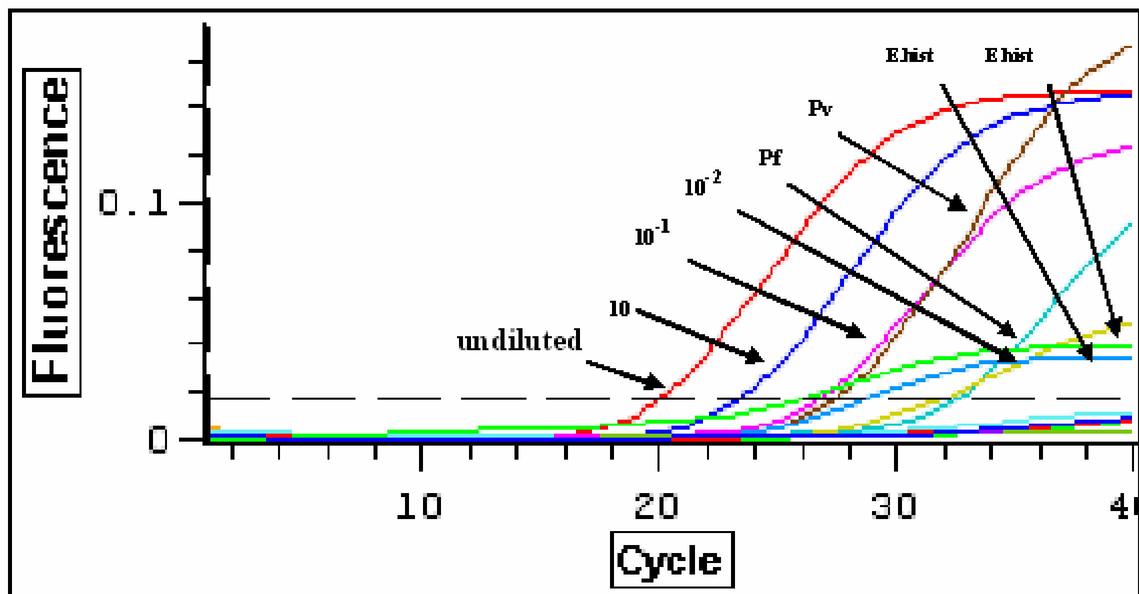

Figure 29 Amplification curves of *Acanthamoeba* spp., *Plasmodium falciparum, Plasmodium vivax* and *Entamoeba histolytica*. DNA from *Acanthamoeba* spp. were serially diluted from 10 ng to 0.001 pg. Amplification curves of all DNA were obtained in all samples by real-time PCR using SYBR Green.

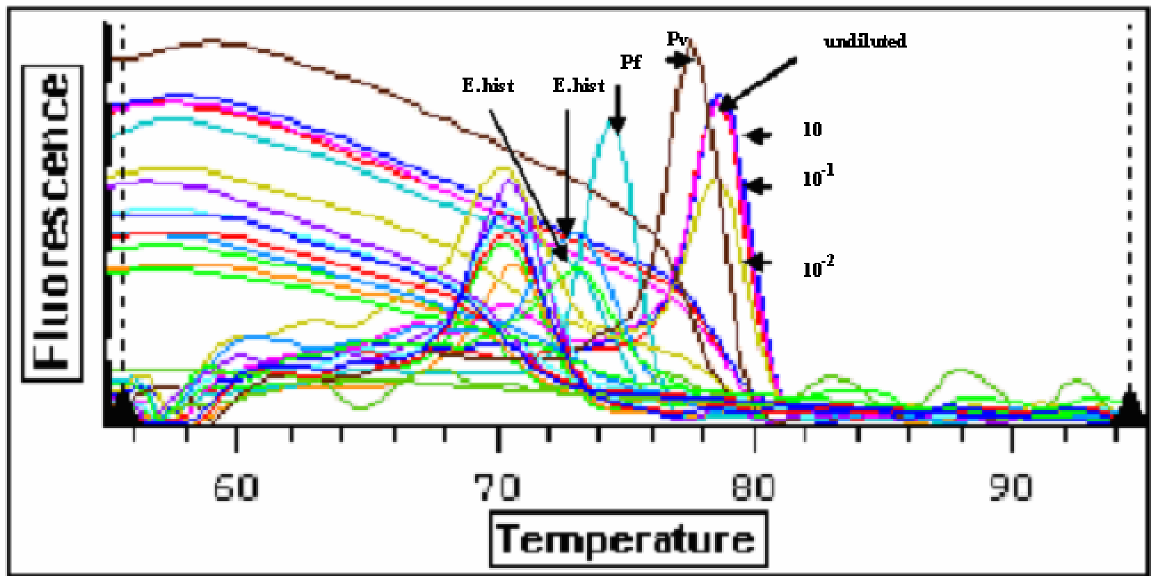

Figure 30 The melting curve analysis of *Acanthamoeba* spp., *Plasmodium falciparum, Plasmodium vivax* and *Entamoeba histolytica*, figure showed melting temperature (T_m) of *Acanthamoeba* spp. was approximately 78.5°C, melting temperature (T_m) of *Plasmodium falciparum* was approximately 74.5°C, melting temperature (T_m) of *Plasmodium vivax* was approximately 77.5°C and melting temperature (T_m) of *Entamoeba histolytica* was approximately 73°C.

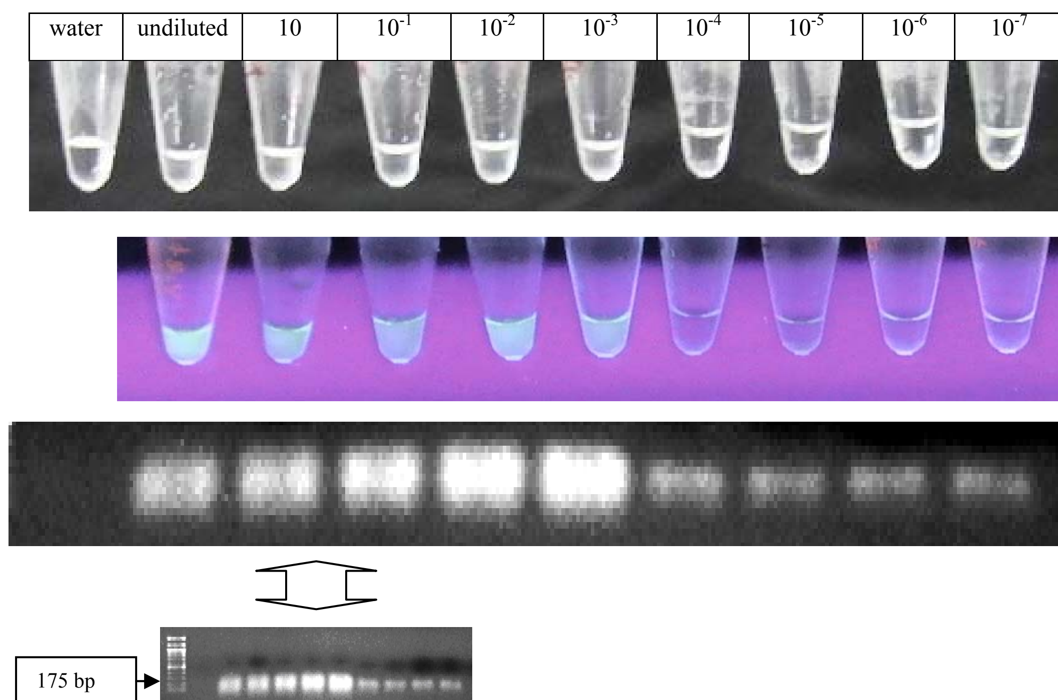

Figure 31 Detection of *Acanthamoeba* spp. by LAMP assays. Total DNAs from *Acanthamoeba* spp. were serially diluted from 10 ng to 0.001 pg and amplified by LAMP after incubation, turbidities of the LAMP reactions were inspected visually. Then confirmed analyzed by Fluorescence dye and electrophoreses in 1.5 % TBE agarose gel. The sizes of the 100 bp size markers are indicated on the left. The results showed that LAMP was able to detect *Acanthamoeba* spp. DNA dilutions up to 10 pg.

Comparison of lower limit of detection in LAMP and Real-time PCR. Total DNAs from *Acanthamoeba* spp. were serially diluted from 10 ng to 0.001 pg and amplified by LAMP and Real-time PCR. F3, B3, FIP and BIP are primer sets used in the LAMP reactions. The F3 and B3 primers in each LAMP primer set were also used in the real-time PCR. The sizes of the 100 bp size markers are indicated on the left.

The results shown LAMP was able to detect *Acanthamoeba* spp. DNA dilutions up to 10 pg and Real-time PCR was able to detect *Acanthamoeba* spp. DNA dilutions up to 100 pg, respectively.

Data analysis

Statistical analysis.

1. Validity of test

The LAMP and culture results from 150 samples will be used for determination of sensitivity and specificity of LAMP results, in this study using culture results as the "gold standard."

Methods	**LAMP**		
Culture		Positive	Negative
	Positive	10	0
	Negative	0	140

Sensitivity = (10/ 10 + 0) × 100

= 100

Specificity = (140/ 140 + 0) × 100

= 100

1. Prevalence Rate

Prevalence Rate = $\frac{10}{150} \times 100$

= 6.7

Table 11 Comparison of methods for the detection of *Acanthamoeba* spp.

Parameter	**Culture**	**Real-time PCR**	**LAMP**
Lower limited detection	ND	100 pg	10 pg
Sensitivity	ND	ND	100
Specificity	ND	ND	100
Time for result	7-14 days	5 hrs	2 hrs
Skill level	High	High	High
Equipment	Incubator Microscopy	Real-time PCR machine	Block heater or water bath
Cost/sample (Baht)*	4.20	255	144

* Only reagent/reaction (September.2007)

The comparison detection methods of *Acanthamoeba* spp. are shown in Table 11. Lower limited detection of Real-time PCR was 100 pg and Lower limited detection of LAMP was 10 pg. Sensitivity and specificity of LAMP was 100%.The three methods can be used to detect positive samples. The speed of diagnosis, LAMP showed less time taken than those other two methods which took approximately 2 hrs. When comparing the cost of each technique, culture was found to be the cheapest method (4.20 Baht/sample) than the other two methods 255 Baht/sample and 144 Baht/sample, respectively.

CHAPTER V
DISCUSSION

Free-living amoebae have the amoeboid, the cystic, and the flagellated forms in their life cycles. The parasitic amoebae are capable of forming cysts as well as free-living aquatic protozoa, especially those living in environmental water, also from cysts to protect themselves from desiccation or other adverse conditions.

Acanthamoeba keratitis has been described primarily from developed countries of the world, with several studies suggesting soft contact lens wear as the greatest risk factor. Contact lens wearers are most at risk from infection of *Acanthamoeba* keratitis cases. Lack of good hygiene to handle the lens, notably the use of non-sterile saline rinsing solutions, and defaulting the practice of the recommended cleaning and disinfection steps are recognized as risk factor. Bacterial biofilm formation on contact lens storage cases may be a risk factor for contact lens associated corneal infection and may explain the persistence of organisms in contact lens storage cases.

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. While waiting for the result from the laboratory which is longs enough for the amoeba to make the infection strikingly worse and more difficult for the treatment to bring recovery. There is an urgent need for rapid diagnosis and prompt treatment especially in case of granulomatous amoebic encephalitis (GAE), otherwise, the victim will die.

In this study we decided to use a molecular diagnostic assay that may have an advantage over cultivation and may be more sensitive and specific, as well as rapid, for the detection of *Acanthamoeba* spp. from contact lens storage cases and to compare these results with culture technique. Loop-mediated isothermal amplification method (LAMP) may provide one answer. A method recently developed by Notomi *et al.* (63) in 2000, relies on autocycling strand displacement DNA synthesis by a *Bst*

DNA polymerase. LAMP requires two specially designed inner and two outer primers as such; LAMP amplifies DNA with high specificity, efficiency, and rapidity under isothermal conditions. Since the LAMP reaction is done under isothermal conditions (65°C), simple incubators, such as a water bath or block heater, are sufficient for the DNA amplification. Moreover, LAMP synthesizes 10 to 20 µg of target DNA within 30 to 60 min, and the LAMP reaction appears to be limited only by amount of deoxynucleoside triphosphates and primers. In the process, a large amount of pyrophosphate ion is produced, which reacts with magnesium ions in the reaction to form magnesium pyrophosphate, a white precipitate by-product. This phenomenon allows easy and rapid visual identification that the target DNA was amplified by LAMP. In this study we designed the LAMP specific primer for amplification of 18S rRNA genes of *Acanthamoeba* spp. The results showed that both of culture-positive specimens were also positive by LAMP too, but LAMP showed more rapid results than culture method. Since the outer primer pair, designated F3 and B3, can also be used for Real-time PCR, the same target gene was amplified from serially diluted total *Acanthamoeba* spp. DNA by both LAMP and Real-time PCR Assay and the sensitivities of lower-limited detection of the two methods were compared.

The observation of the proper temperature for *Acanthamoeba* spp. growth in contact lens storage cases and cultivation of *Acanthamoeba* spp. on non-nutrient agar.

This study has demonstrated that the bacterial biofilm phenomenon in contact lens storage cases is common in wearers with bacterial and *Acanthamoeba* keratitis, despite good compliance with manufacturer's instructions for their lens cleaning system. Biofilm formation on the contact lens storage cases may also provide one explanation for the development of keratitis in wearers of disposable extended wear lenses. Organisms in a sessile mode of growth in a low nutrient environment may be dormant and not easily isolated by conventional culture techniques. The use of low nutrient media may result in a higher culture positive rate. Biofilm was found more frequently and more densely in contact lens storage cases. These may imply that the contact lens storage cases are the initial source of contamination and contact lens storage cases contamination occurs subsequently. Once contact lens storage cases

colonization and biofilm formation has occurred, plank tonic bacteria are likely to shed from the body of the biofilm and will be attracted to the contact lens. The contact lens storage cases may present a more favorable environment for micro-organism colonization and proliferation and any micro-organisms adhering to the contact lens may be removed during wearing of contact lens. The contact lens storage cases are a static, relatively low nutrient environment, which is likely to be ideal for biofilm development. In addition, the design and condition of the contact lens storage cases may provide an ideal niche for bacterial colonization and biofilm development, which resists normal hygiene methods. It is possible that growth within contact lens storage cases biofilm is advantageous for the survival of *Acanthamoeba* spp..

Acanthamoeba keratitis in contact lens wearers is frequently associated with bacterial biofilm in the contact lens storage cases. Despite the use of current contact lens disinfection systems, the contact lens storage cases are a favorable environment for proliferation of certain organisms. Biofilm on the contact lens storage cases may prolong the retention of organisms at the ocular surface and increase their potential pathogenicity.

The results of *Acanthamoeba* spp. detection by Loop-mediated isothermal amplification of DNA (LAMP).

Optimized conditions for LAMP

The sequences and sizes of the primers were chosen so that their melting temperatures (T_m) fell within certain ranges. The F2 and B2 sequences in FIP and BIP were chosen such that their T_m values fell between 60 and 65°C, the optimal temperature for *Bst* polymerase. The T_m values of F1c and B1c were set slightly higher than those of F2 and B2 in order that a looped out structure formed immediately after release of the single stranded DNA from the template. Furthermore, the T_m values of the outer primers (F3 and B3) were set lower than those of F2 and B2 in order to ensure that synthesis occurred earlier from the inner primers than from the outer primers. In addition, the outer primers were used at 1/4-1/10 the concentration of inner primers.

The efficiency of LAMP depends on the size of the target DNA because one rate limiting step for amplification in this method is strand displacement DNA

synthesis. Therefore, the size of target DNA should be set to less than 300 bp, including F2 and B2.

The visual identification must be performed in 10 minutes after terminating the reactions because the turbidity of reactions was clearly appear. For gel electrophoresis, its could be better indicated the results than visual identification because in some cases, a white precipitate by-product reaction form magnesium pyrophosphate less than could be detection by visual.

The magnesium was important for reaction because it function of cofactor to dNTP to amplify DNA in LAMP reactions.

Comparison of the lower limit of detection in LAMP and Real-time PCR Assay.

Since the outer primer pair, designated F3 and B3, can also be used for Real-time PCR, the same target gene was amplified from serially diluted total *Acanthamoeba* spp. DNA by both LAMP and Real-time PCR Assay and the sensitivities of lower-limited detection of the two methods were compared. The results showed that LAMP was able to detect *Acanthamoeba* spp. DNA dilutions up to10 pg and Real-time PCR was able to detect *Acanthamoeba* spp. DNA dilutions up to 100 pg.

These results also suggest that, LAMP can be used for detection of *Acanthamoeba* spp., it does not require thermal cyclers, or skilled technicians. Moreover, LAMP requires only a simple incubator, such as a block heater or water bath that provides a constant 60-65°C, which makes it more economical and practical than real-time PCR. The white turbidity of magnesium pyrophosphate accumulation as by-product of DNA amplification can be detected by the naked eye or by turbidimeter and should be detected in 10 sec after terminated of the reactions. Alternatively, amplification can be simply inspected by naked eye using fluorescence, which turns green in the presence of amplified DNA. Fluorescence results were consistent with those deduced from the real-time turbidimeter. Since the turbidity assay can be carried out in a closed system, the risk of contamination is lower than that of agarose gel electrophoresis. This is an additional advantage of LAMP for clinical use.

CHAPTER VI
CONCLUSION

Acanthamoeba spp. are ubiquitous free-living protozoa found in a wind range of environmental niches. They are resistant to disinfectants, temperature variation, desiccation and they are etiologic agents of granulomatous amoebic encephalitis (GAE), *Acanthamoeba* keratitis (AK), skin lesions and peptic ulcer. This study 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 users.

The main objectives of this study were the detection of *Acanthamoeba* from contact lens cases by culture method on non-nutrient agar (NNA) and compared these results with the Loop-mediated isothermal amplification method (LAMP) and comparison of low limited detection in LAMP and Real-time PCR and survey the prevalence of *Acanthamoeba* spp. in contact lens wearers in student at Silapakorn University.

This study was conducted at Silapakorn University, Nakhornpathom Province. Laboratoties were performed at the Faculty of Public Health, Department of Parasitology, Mahidol University, Bangkok. This study was performed since March till October 2007.

In this study, the prevalence of *Acanthamoeba* spp. in contact lens wearers of student at Silapakorn University was 6.7 % (10/150). Cysts of the isolated amoebae were studied morphologically and devided into 3 different subgenera according to the characteristics previously described by Tsuruhara.

The results of *Acanthamoeba* spp. cultivation in contact lens storage cases and on non-nutrient agar shown that the *Acanthamoeba* spp. could be grown at 36.8-37.2°C and biofilm was found more frequently and more densely in contact lens storage cases. This may imply that the contact lens storage cases are the initial source for contamination and contact lens storage cases contamination occurs subsequently.

Acanthamoeba keratitis in contact lens wearers is frequently associated with bacterial biofilm in the contact lens storage cases. Despite the use of current contact lens disinfection systems, the contact lens storage cases are a favorable environment for proliferation of certain organisms. Biofilm on the contact lens storage cases may prolong the retention of organisms at the ocular surface and increase their potential pathogenicity.

The results of *Acanthamoeba* spp. detection by Loop-mediated isothermal amplification of DNA (LAMP) shown both of cultures positive were also positive by LAMP too, but LAMP shown more rapid results than culture. The temperatures of reactions were chosen so that their melting temperatures (T_m). The F2 and B2 sequences in FIP and BIP were chosen such that their T_m values fell between 60 and 65°C, the optimal temperature for *Bst* polymerase. The visual identification must be performed in 10 minuted after terminated of the reactions because the turbidity of reactions were clearly appear and the size of DNA products were 175 bp.

Comparison of the lower limit of detection in LAMP and Real-time PCR Assay. The results shown LAMP was able to detect *Acanthamoeba* spp. DNA dilutions up to10 pg and Real-time PCR was able to detect *Acanthamoeba* spp. DNA dilutions up to 100 pg. The LAMP methods developed in this study can be useful for clinical diagnosis. Compared to culture and real-time PCR, LAMP has the advantages of reaction simplicity and detection sensitivity. LAMP does not require complicated thermal cycling step; an isothermal reaction for a rather short time (about 1 hour) is enough to amplify the target DNA to detectable levels. Another useful feature of LAMP lies in the opportunity for turbidity-based detection of the positive reaction. The turbidity of the LAMP reaction mix can be easily judged by the naked eye. Because real-time PCR and other molecular biological techniques are best conducted only in well-equipped laboratories, these methodologies are often impracticable under conditions requiring a field diagnosis. In contrast, the useful characteristics of LAMP that we have described make it possible to use this highly sensitive DNA amplification method in many places, under field conditions and in local clinics and hospitals where cost and environmental restraints prohibiting Real-time PCR are otherwise in effect. While we have taken an important first step, further

improvements are still needed,i.e., with our current primers, LAMP detects both *Acanthamoeba* spp..

In the future LAMP shall be used as the rapid diagnosis for *Acanthamoeba* keratitis. These techniques are more sensitive than the conventional methods, since DNA amplifications can be achieved from the small number of DNA. We hope LAMP will still be useful for the initial screening of suspected infection caused by *Acanthamoeba* spp., important causative agents of *Acanthamoeba* keratitis in contact lens wearers.

Prevention of persistent contamination and colonization of the contact lens storage cases may help to reduce the incidence of *Acanthamoeba* keratitis in contact lens wearers. Strategies such as modifying the contact lens storage cases materials or their coatings to prevent adherence and colonization, the use of anti-adhesive agents, or altering the formulation of care systems to improve the penetration of disinfecting agents into biofilms, may be helpful. The contact lens storage cases care systems must be tested and proved effective against bacteria in the biofilm mode of growth before being licensed for use. In addition to these potential strategies, improved contact lens wearer education, compliance with contact lens storage cases, hygiene recommendations and regular contact lens storage cases disposal may help to limit the problem.

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APPENDIX

APPENDIX A

Biological Media

Page's amoeba saline (10x)

Sodium chloride (NaCl)	1.20 g
Magnesium sulfate ($MgSO_4.7H_2O$)	0.04 g
Sodium phosphate, dibasic (Na_4 HPO_4)	1.42 g
Potassium phosphate, monobasic (KH_4 PO_4)	1.36 g
Calcium chloride ($CaCl_2.2H_2O$)	0.04 g
Double distilled water to	1,000 ml

Sterilize by autoclaving at 121°C for 15 min.

Cool and store at 4°C. An expiration date of 3 months.

1.5% Nonnutrient agar (NNA)

10x amoeba saline	100.0 ml
Difco agar	15.0 g
Double-distilled water	900.0 ml

Autoclave at 121°C for 15 min, Cool to about 60°C and aseptically pour into sterile plastic petri dishes, store the agar plates in canisters at 4°C and an expiration date of 3 months.

APPENDIX B

Results record form

Sample No.	Results	
	LAMP	Culture (Gold Standard)
1	Negative	Negative
2	Negative	Negative
3	Negative	Negative
4	Negative	Negative
5	Negative	Negative
6	Negative	Negative
7	Negative	Negative
8	Negative	Negative
9	Negative	Negative
10	Negative	Negative
11	Negative	Negative
12	Negative	Negative
13	Negative	Negative
14	Negative	Negative
15	Negative	Negative
16	Negative	Negative
17	Negative	Negative
18	Negative	Negative
19	Negative	Negative
20	Negative	Negative
21	Negative	Negative

Sample No.	**Results**	
	LAMP	**Culture (Gold Standard)**
22	Negative	Negative
23	Negative	Negative
24	Negative	Negative
25	Negative	Negative
26	Negative	Negative
27	Negative	Negative
28	Negative	Negative
29	Negative	Negative
30	Negative	Negative
31	Negative	Negative
32	Negative	Negative
33	Negative	Negative
34	Negative	Negative
35	Negative	Negative
36	Negative	Negative
37	Negative	Negative
38	Negative	Negative
39	Negative	Negative
40	Negative	Negative
41	Negative	Negative
42	Negative	Negative

Sample No.	**Results**	
	LAMP	**Culture (Gold Standard)**
43	Negative	Negative
44	Negative	Negative
45	Negative	Negative
46	Negative	Negative
47	Negative	Negative
48	Negative	Negative
49	Negative	Negative
50	Negative	Negative
51	Negative	Negative
52	Negative	Negative
53	Negative	Negative
54	Negative	Negative
55	Negative	Negative
56	Negative	Negative
57	Negative	Negative
58	Negative	Negative
59	Negative	Negative
60	Negative	Negative
61	Negative	Negative
62	Negative	Negative
63	Negative	Negative

Sample No.	Results	
	LAMP	Culture (Gold Standard)
64	Negative	Negative
65	Negative	Negative
66	Negative	Negative
67	Negative	Negative
68	Negative	Negative
69	Negative	Negative
70	Negative	Negative
71	Negative	Negative
72	Negative	Negative
73	Negative	Negative
74	Negative	Negative
75	Negative	Negative
76	Negative	Negative
77	Negative	Negative
78	Negative	Negative
79	Negative	Negative
80	Negative	Negative
1A	Negative	Negative
2A	Negative	Negative
3A	Negative	Negative

Sample No.	Results	
	LAMP	Culture (Gold Standard)
4A	**Positive**	**Positive**
5A	**Positive**	**Positive**
6A	Negative	Negative
7A	Negative	Negative
8A	**Positive**	**Positive**
9A	Negative	Negative
10A	**Positive**	**Positive**
11A	Negative	Negative
12A	Negative	Negative
13A	Negative	Negative
14A	Negative	Negative
15A	Negative	Negative
16A	Negative	Negative
17A	Negative	Negative
18A	Negative	Negative
19A	Negative	Negative
20A	Negative	Negative
21A	Negative	Negative
22A	Negative	Negative
23A	Negative	Negative
24A	Negative	Negative

Sample No.	**Results**	
	LAMP	**Culture (Gold Standard)**
25A	Negative	Negative
26A	Negative	Negative
27A	Negative	Negative
28A	Negative	Negative
29A	Negative	Negative
30A	Negative	Negative
31A	Negative	Negative
32A	Negative	Negative
33A	Negative	Negative
34A	Negative	Negative
35A	**Positive**	**Positive**
36A	**Positive**	**Positive**
37A	Negative	Negative
38A	Negative	Negative
39A	Negative	Negative
40A	Negative	Negative
41A	Negative	Negative
42A	**Positive**	**Positive**
43A	Negative	Negative
44A	Negative	Negative
45A	**Positive**	**Positive**

Sample No.	Results	
	LAMP	Culture (Gold Standard)
46A	Negative	Negative
47A	Negative	Negative
48A	Negative	Negative
49A	**Positive**	**Positive**
50A	**Positive**	**Positive**
51A	Negative	Negative
52A	Negative	Negative
53A	Negative	Negative
54A	Negative	Negative
55A	Negative	Negative
56A	Negative	Negative
57A	Negative	Negative
58A	Negative	Negative
59A	Negative	Negative
60A	Negative	Negative
61A	Negative	Negative
62A	Negative	Negative
63A	Negative	Negative
64A	Negative	Negative
65A	Negative	Negative
66A	Negative	Negative
67A	Negative	Negative
68A	Negative	Negative
69A	Negative	Negative
70A	Negative	Negative

BIOGRAPHY

NAME	Miss Rangsima Passara
DATE OF BIRTH	8 November 1979
PLACE OF BIRTH	Songkhla Province, Thailand
INSTITUTIONS ATTENDED	Mahidol University 1998-2001
	Bachelor of Nursing
	Mahidol University 2004 -2007
	Master of Science (Public Health)
	Major in Infectious Diseases and
	Epidemiology
POSITION & OFFICE	2003 - Present
	Position: Nurse
	Office: Nonthavej Hospital
	Ngamwongwan road
	Nonthaburi, Thailand
HOME ADDRESS	86 M.10 Tambon Khutoa
	Amphur Hatyai
	Songkhla Province, Thailand
RESEARCH GRANT	Supported in part by the Thesis Grant,
	Faculty of Graduate Studies, Mahidol
	University)