國立臺灣大學公共衛生學院環境衛生研究所

碩士論文

Graduate Institute of Environmental Health

College of Public Health

National Taiwan University

Master Thesis

泳池與農田棘阿米巴原蟲定性研究

Characterization of *Acanthamoeba* Isolated from Swimming Pools and Farmlands

張維珍

Wei-Chen Chang

指導教授：張靜文 博士

Advisor: Ching-Wen Chang, *Ph.D.*

中華民國一百零一年七月

July, 2012

ACKNOWLEDGEMENTS

The research works of this dissertation hold far more than the culmination of three years of study. Completing the thesis is a challenge, thanking all those who contributed to it is an even greater one. These pages also reflect the relationships with many generous and inspiring people I have met since beginning my graduate work. Due to the nature of my study, many people were involved in one or the other way in it. I want to thank all of them, including those not mentioned here by name.

First and foremost I want to thank my advisor Ching-Wen Chang. Her constant encouragement, support, and invaluable suggestions made this work successful. She has taught me, both consciously and unconsciously, how good experimental is done. I appreciate all her contributions of time and ideas.

I would also like to thank the members of my committee to attend my defense: Dr. Chien-Shien Chen, Dr, Bing-Mu Hsu and Dr. Dar-Der Ji for their encouraging words, thoughtful criticism, and time. Their advice and patience is appreciated.

I am deeply indebted to Dr. Dar-Der Ji and Jung-Sheng Chen for their help and valuable advice throughout my research. Without their help, this work would not be possible.

Additionally, I would like to thank all the members of my research group for their laboratory assistance, advice, and friendship. I am also indebted to the fellow graduate students before me.

The members of the Institute of Environmental Health at National Taiwan University were helpful to me in my research and for sharing their enthusiasm and comments on my work. To my invaluable supportive, forgiving, generous and loving friends without whom I could not have survived the process.

Lastly, for my parents, who raised me with a love of science and supported me in all my pursuits. I am deeply and forever indebted to my parents for their love, support, encouragement throughout my entire life and understanding during the long years of my education. I thank you for being with me in the sun and in the shadow.

Finally, thank you to my family, friends, and especially my family, for their encouragement, constant support, and patience as I've followed my own path.

Wei-Chen Chang

National Taiwan University

July 2012

摘要

棘阿米巴原蟲（*Acanthamoeba*）為自營性阿米巴原蟲，廣泛存在於自然及人工環境的水體、土壤與空氣中。棘阿米巴原蟲為伺機性的致病微生物，可導致嚴重威脅視力的棘阿米巴角膜炎、以及九成高致死率的肉芽腫性阿米巴腦膜炎。棘阿米巴原蟲感染的機制取決於棘阿米巴原蟲致病性、宿主與環境因子。此外，棘阿米巴原蟲不只感染人類，也是其他致病菌的自然界宿主。

本研究利用形態學和分子生物學方法檢測來自台北市 6 個游泳池之 12 件游泳池樣本、以及來自 6 個屏東縣恆春鎮和 5 個屏東縣車城鎮洋蔥田農田之 13 件土壤與 14 件空氣樣本，鑑定其中是否有棘阿米巴原蟲，並利用基因型分析、耐熱性及耐滲透壓測試，評估分離的棘阿米巴原蟲是否具潛在致病性。另收集水體 pH、水溫、導電度、濁度、硬度、溶氧、餘氯、鹽度、總溶解固體及異營性總細菌濃度，測量土壤 pH 和、含含量及異營性總細菌濃度，以及採樣時空氣溫度、風速和相對濕度，將環境因子特性與棘阿米巴原蟲基因型、耐熱及耐滲透壓結果交叉比較，分析環境因子是否影響棘阿米巴原蟲之潛在致病性。

結果發現，6 個游泳池水樣檢出率為 100%，13 件屏東縣洋蔥田土壤檢出率亦為 100%，其中 14 件空氣樣本中只有 3 件檢出棘阿米巴原蟲，其檢出率為 21.43%。本研究共分離 42 株棘阿米巴原蟲，其中泳池水樣分離出 26 株、土壤為 13 株、空

氣則是 3 株。在泳池水樣的 26 株皆為 *Acanthamoeba polyphaga*，且皆屬於基因型 T4。其中 12 株進行耐熱性和滲透壓測試，耐熱結果顯示除了內湖運動中心分離的一株不能耐熱 37^{o}C 外，其餘 11 株都可耐熱 37^{o}C，但此 12 株都無法耐熱於 42^{o}C 及 52^{o}C 下；至於滲透壓結果顯示，12 株泳池分離菌株在 1M mannitol 的培養基上都能生長。

至於在土壤分離之 13 株棘阿米巴原蟲，6 株為 *Acanthamoeba polyphaga*，屬於基因型 T4，另外 7 株為 *Acanthamoeba lenticulata*，屬於基因型 T5。將此 13 株進行耐熱性和滲透壓測試，結果顯示 7 株可耐熱於 52^{o}C，2 株耐熱於 42^{o}C，4 株則僅能耐熱至 37^{o}C；滲透壓結果顯示所有 13 株皆無法生長在 1 M mannitol 的培養基，但都能在 0.5 M mannitol 的培養基中生長。

對於分離自空氣樣本的 3 株棘阿米巴原蟲，2 株為 *Acanthamoeba polyphaga*，屬於基因型 T4，另外 1 株為 *Acanthamoeba* species，屬於基因型 T15。將此 3 株進行耐熱性和滲透壓測試，結果顯示，除恆春農田分離的一株棘阿米巴原蟲不能耐熱 37^{o}C 外，另 2 株均可耐熱 37^{o}C，但此 3 株都不能耐熱至 42^{o}C 與 52^{o}C；滲透壓結果顯示，有一株棘阿米巴原蟲能在 0.5 M mannitol 與 1M mannitol 下生長，一株棘阿米巴原蟲僅能生長於 0.5 M mannitol 的培養基，一株則無法在 0.5 M mannitol 和 1 M mannitol 的培養基上生長。

依據基因型、耐熱性和滲透壓結果顯示，泳池分離出來的棘阿米巴原蟲均屬

T4，且一株不能在 37°C 生長，其餘均能在 37°C 與滲透壓 1 M mannitol 下生長；而農田土壤及空氣分離之 16 株棘阿米巴原蟲大多數無法在 1M mannitol 的環境下生長，這顯示泳池中之棘阿米巴原蟲一旦有機會接觸到人體的眼睛，多可忍受人體體溫與淚液之高滲漏壓。意謂泳池內棘阿米巴原蟲可能具有眼睛角膜之致病風險，然土壤分離之部分菌株可耐高溫，仍宜關切其潛在風險。

關鍵字：棘阿米巴原蟲、角膜炎、肉芽腫性阿米巴腦膜炎、致病性、PCR、環境因子、生物指標、基因型、耐熱性、耐滲透壓

ABSTRACT

Acanthamoeba is free-living amoebae and ubiquitous in a wide variety of natural habitats and human-made environments, including water, soil, and air, and a opportunistically pathogenic organism that can cause a severe sight-threatening *Acanthamoeba* keratitis (AK), and a fatal infection of the central nervous system (granulomatous amoebic encephalitis, GAE), mortality rate can up to 90%. The mechanisms associated with the pathogenesis of *Acanthamoeba* tend to be highly complex, depending on parasite, host and the environmental factors. Besides, these amoebae are not only infective to human but also the hosts for other pathogenic bacteria to multiply in the environments.

In this study, we isolated and characterized *Acanthamoeba* from water, soils, and air in the effort to determine the presence and potentially pathogenic *Acanthamoeba.* There were 12 water samples from 6 chlorinated swimming pools in Taipei city, 13 soil samples and 14 air samples were from 6 onion farmlands in Hengchun Township, Pingtung County and 5 onion farmlands in Checheng Township, Pingtung County. The samples were then followed by morphology and molecular identification, phylogenetic and physiologically assays including thermotolerance and osmotolerance

characterization. Environmental factors in water samples including pH, water temperature, conductivity, turbidity, hardness, dissolved oxygen, free chlorine, salinity, total dissolved solids and heterotrophic plate count. Water content, temperature and heterotrophic plate count in soil samples. Temperature, wind velocity and relative humidity in air were measured in order to characterize the sampling sites and to determine how these factors affecting the potentially pathogenicity of isolated *Acanthamoeba* by genotyping, thermotolerance and osmotolerance.

In the present study, we discovered *Acanthamoeba* in six swimming pools (100%), 13 onion farmlands (100%), and only three out of 14 air samples (21.43%). Total 42 isolates of *Acanthamoeba* are isolated, 26 isolates from swimming pools, 13 isolates from soil, and three isolates from air samples. The 26 isolates in swimming pools are belong to *Acanthamoeba polyphaga*, and genotype T4, there are 12 isolates taken into thermotolerance and osmotolerance among all, only one isolate from NEIHU swimming pool cannot growth under 37^{o}C, and 12 isolates cannot growth under 42^{o}C and 52^{o}C, in osmotolerance, 12 isolates can tolerant and growth up to 1 M mannitol.

In 13 soil isolates, six isolates belong to *Acanthamoeba polyphaga* and genotype T4, and the rest 7 isolates belong to *Acanthamoeba lenticulata*, genotype T5, all 13 isolates were testing for the thermotolerance and osmotolerance, 7 isolates can tolerant

up to 52^{o}C, two isolates growth under 42^{o}C, and only four isolates growth at 37^{o}C, and 13 isolates cannot growth at 1 M mannitol, but can growth at 0.5 M mannitol.

In three air isolates, two isolates belong to *Acanthamoeba polyphaga* and genotype T4, one isolate belongs to *Acanthamoeba* species, genotype T15, all three isolates were testing for the thermotolerance and osmotolerance, the 2 isolates can growth at 37^{o}C besides isolate in HENGCHUN onion farmlands 4, which cannot growth at 37^{o}C, three isolates cannot growth under 42^{o}C and 52^{o}C, when it comes to osmotolerance, only one isolate can growth under 0.5 M mannitol and 1 M mannitol, one isolate can growth under 0.5 M mannitol, and the other isolate cannot growth neither 1 M mannitol nor 0.5 M mannitol.

The pathogenicity based on genotyping, thermotolerance and osmotolerance revealed the all isolates from swimming pools in present study are genotype T4 and among the 12 isolates with thermotolerance and osmotolerance, 11 isolates can grew at 37^{o}C and 1 M mannitol. Once the pool isolates contact with human eyes, they can tolerate body temperatures and osmolarity of tear film. Most of 16 isolates from soil and air samples indicated weak pathogenicity since these isolates cannot growth under 1 M mannitol. *Acanthamoeba* isolates from swimming pools indicated higher potentially pathogenicity while the potential risk on isolates from soils since isolates can withstand

at higher temperature.

Key word: *Acanthamoeba*, keratitis, granulomatous amebic encephalitis, pathogenicity, PCR, environmental factors, biological indicator, genotype, thermotolerance, osmotolerance

CONTENTS

LIST OF TABLES

LIST OF FIGURS

ABBREVIATIONS

Abbreviation	Full Name
AIDS	Acquired Immunodeficiency Syndrome
AK	*Acanthamoeba* Keratitis
ANS	*Acanthamoeba*-Negative Samples
APHA	American Public Health Association
APS	*Acanthamoeba*-Positive Samples
ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
CCAP	Culture Collection of Algae and Protozoa
CFU	Colony-Fosrming Units
CNS	Central Nervous System
Ct	Cycle Threshold
DNA	DeoxyriboNucleic Acid
FLA	Free-Living Amoebae
GAE	Granulomatous Amebic Encephalitis
HIV	Human Immunodeficiency Virus
HPC	Heterotrophic Plate Count
LDH	Lactate Dehydrogenase
MEGA	Molecular Evolutionary Genetics Analysis
MK	Microbial Keratitis
NA	Nutrient Agar
NNA	Non-Nutrient Agar
PAS	Page's Amoeba Saline
PCR	Polymerase Chain Reaction
PYG	Peptone Yeast Glucose
RH	Relative Humidity
SP	Swimming Pool
TDS	Total Dissolved Solids

Chapter 1 Introduction

1.1 Background

Acanthamoeba are free-living amoebae (FLA), ubiquitous organisms and have been isolated from soil, water (including natural and treated water), air, and dust. They are opportunistic pathogens, not only can cause painful, sight-threatening keratitis as well as fatal infections, also act as carriers and reservoirs for bacteria such as *Legionella* spp. to result in serious diseases on human health. *Acanthamoeba* infections have become increasingly important since the increasing populations of contact lens users and acquired Immunodeficiency Syndrome, AIDS patients. The significance of health issue implicated by *Acanthamoeba*, the distribution and pathogenicity of *Acanthamoeba* in the environments are highly important in the rational to develop well therapeutic interventions since successful treatment requires early and proper diagnosis.

1.2 Literature Review

1.2.1 Biology and distribution of *Acanthamoeba*

Acanthamoeba are ubiquitous and among the most prevalent protozoa discovered in the environment (Mergeryan, 1991; Page, 1967; Rivera et al., 1991; Rodriguez-Zaragoza, 1994). They have been isolated from swimming pools (Gornik et al., 2004; Gianinazzi et al., 2009; Janitschke et

al., 1980; Lyons et al., 1977; Rivera et al., 1983; Rivera et al., 1993; Vesaluoma et al., 1995), tap water (Kilvington et al. 2004; Rivera et al., 1979), spa (Martinez, 1985; Rivera et al., 1987), hot spring (Hsu et al., 2009), soils (Kawaguchi et al., 2009; Lorenzo-Morales et al., 2005; Rezaeian et al., 2008; Tsvetkova et al., 2004) and air (Rivera et al., 1987; Rivera et al., 1994; Rogerson and detwiler, 1999). The life cycle of *Acanthamoeba* undergoes two stages: an actively feeding and dividing trophozoite and a dormant cyst. The trophozoite varies in size ranging from 25 to 40 μm and actively feed on bacteria, algae, yeasts or small organic particles (Bowers, 1977; Bowers and Olszewski, 1983). Cell division is asexual and occurs by binary fission. However, the transformation of trophozoite into cyst stage occurs under harsh conditions such as food deprivation, desiccation, increased osmolarity and extremes in temperatures and pH (Bowers and Korn, 1969; Byers et al., 1980; Chagla and Griffiths, 1974). A double-walled wrinkled cyst composed of an ectocyst and an endocyst with the size ranging from 13 to 20 μm (Bowers and Korn, 1969). Cysts are highly resistant to harsh conditions such as low temperatures (0 to 2°C) (Brown and Cursons, 1977), high temperatures (up to 80°C) (Storey et al., 2004), biocides (Lee et al., 2007; Lloyd et al., 2001; Khunkitti et al., 1997;), chlorination (De Jonckheere et al., 1976; King et al., 1988; Kilvington et al., 1990), and antibiotics (De Jonckheere and Van de Voorde, 1976; Khunkitti et al., 1998; Lloyd et al., 2001; Turner et al., 2000). De Jonckheere et al. (1991) indicated that *Acanthamoeba* can withstand the extremes in temperature, desiccation and disinfection, which also

correlates well with the high frequency of their isolations from environments. Besides, cysts can remain viable for several years while maintaining their pathogenicity (Mazur et al., 1995).

1.2.2 *Acanthamoeba* as human pathogens

1.2.2.1. Granulomatous Amebic Encephalitis (GAE)

The first clearly identified Granulomatous Amebic Encephalitis (GAE) in humans was observed by Jager and Stamm (1972). GAE, as an opportunistic disease, is characterized by a chronic protracted, slowly progressive central nervous system (CNS) infection. GAE occurs in the host whose metabolic, physiological, or immunological integrity are compromised, and it is generally associated with the individuals who already have underlying diseases such as human immunodeficiency virus (HIV) infection or Hodgkin's disease (Jager et al., 1972; Martinez et al., 1982; Martinez et al., 1997; Steinberg et al., 2002). Although an enhanced susceptibility to GAE infection is associated with immune suppression, cases of GAE caused by *Acanthamoeba* have been found in immunecompetent children and adults (Ofori-Kwakye et al., 1986; Ringsted et al., 1976; Sangruchi et al., 1994; Singhal et al., 2001).

There are two possible routes for *Acanthamoeba* to enter human CNS: by inhalation of *Acanthamoeba* through the nasal passage and lung or by the blood. Skin lesions may provide direct entry of *Acanthamoeba* into the bloodstream, bypass the lower respiratory tract. Entry of

Acanthamoeba into the CNS is most likely occurred through the blood-brain barrier (Martinez, 1985; Martinez, 1991).

In Taiwan, the first case of GAE caused by *Acanthamoeba castellanii* occurs in a previously healthy farmer (Liang et al., 2010; Sheng et al., 2009). The significance of these studies was to show the presence of clinically relevant amphizoic amoebae in vegetated farmlands, which may present a risk to farmers' health. The risk factors of GAE infection is of major concern in view of (i) increasing number of immunocompromised persons, (ii) more individuals undergoing immunosuppressive therapy and excessive use of steroids, and (iii) the global warming which may add to the ubiquity of these pathogens, and thus a possibility of increased exposure to the susceptible hosts (Khan, 2008).

1.2.2.2. *Acanthamoeba* keratitis (AK)

Acanthamoeba keratitis (AK) has become increasingly recognized as important in human health. AK was first reported by Nagington et al. (1974) in the United Kingdom. Associations of AK with ocular trauma or contact with contaminated water by Jones et al. (1975) in the United States.

Incidence rate of AK varies from geographical locations. An incidence rate of 0.33 per 10,000 contact lens wearers was reported in Hong Kong (Houang et al., 2001), 0.05 per 10 000 in Holland

(Cheng et al., 1999), 0.01 per 10 000 in the USA (Stehr-Green et al., 1989), 0.19 per 10 000 in England (Radford et al., 2002) and 1.49 per 10 000 in Scotland (Lam et al., 2002; Seal et al., 1999). However, variations in incidence rate do not reflect the geographical distribution of *Acanthamoeba*, and are most likely due to extended wear of soft contact lenses, lack of awareness of the potential risks associated with wearing contact lenses, enhanced detection, and/or environmental conditions that promote growth of pathogenic amoebae, e.g. higher water hardness (Radford et al., 2002).

Table 1 indicates the prevalence rate of microbial keratitis, MK caused by *Acanthamoeba,* the highest prevalence rate is 31.0%, observed in England and Wales (Radford et al., 2002), followed by 26.9% in West of Scotland, UK (Seal et al., 1999), and 13.9% in Taiwan (Chen et al., 2004). High prevalence rate of microbial keratitis caused by *Acanthamoeba* in Taiwan indicates AK is an important issue that threatened Taiwanese health.

Table 1 Prevalence of microbial keratitis caused by *Acanthamoeba* and the risk factors in worldwide

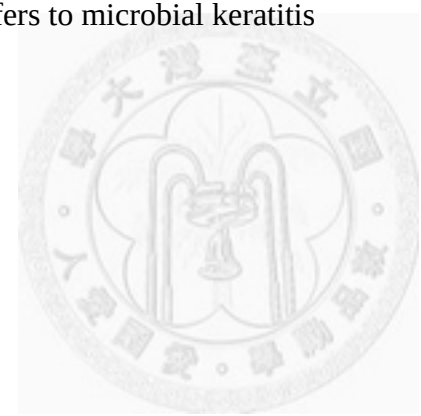
Country	Location	Study year	AK/MK (%)[a]	Risk factors for MK	Reference
England and Wales	The Royal College of ophthalmologists set up by the British Ophthalmic Surveillance Unit (BOSU)	Two years' data collection commenced on 1 October 1997	33/106 (31.0%)	Non-contact lens wearers: 77.8% for a history of minor ocular trauma and/or eyes having been splashed 11.1% for regularly splashed water into eyes 11.1% for oncurrent uveitis and glaucoma, used an eye bath Contact lens wearers: 8% for a history of trauma 3% for a medical indication 6% for concurrent eye disease	Radford et al., 2002
West of Scotland, UK	The Glasgow Eye and Western Infirmaries	May 1st to December 31st, 1995	14/52 (26.9%)	Either of previous chronic ocular surface disease or contact lens wear	Seal et al., 1999
Taiwan	NTU Hospital, Taipei	1987 to 2001	15 / 108 (13.9%)	38.6% for ocular trauma 27.3% for contact lens wear	Chen et al., 2004

Hong Kong	Hong Kong Eye Hospital (HKEH) and the Prince of Wales Hospital (PWH)	April 1997 to August 1998	6/90 (6.7%)	Non-contact lens wearers: 48% for previous eye disease 40% for use of antibiotic prior to hospital referral 27% for history of trauma 7% for use of steroid prior to hospital referral Contact lens wearers: 58% for use of antibiotic prior to hospital referral 7% for previous eye disease 5% for history of trauma 3% for use of steroid prior to hospital referral	Lam et al., 2002
Ireland	The Royal Victoria Eye and Ear Hospital (RVEEH), Dublin, Ireland	September 2001 and August 2003	2/90 (6.1%)	41.1 for contact lens wear 21.1 for anterior segment disease 14.4% for ocular trauma 4.4% for systemic disease 1.1% for previous ocular surgery 16.7% were no risk factor identified	Saeed et al., 2009
Taiwan	Chang Gung Memorial Hospital	2000 to 2003	1/20 (5%)	Overnight orthokeratology	Hsiao et al., 2005
Australia	Sydney Eye Hospital	January 1997 and December 2002	20/426 (4.7%)	80% for contact lenses 40% for additional risk factors including poor lens hygiene	Butler et al., 2005

Taiwan	Hsin-Chu Hospital, Hsin-Chu; and NTU Hospital, Taipei	1992 to 2001	22 / 504 (4.4%)	44.3% for contact lens wear 23.8% for ocular trauma	Fong et al., 2004
Taiwan	Chang Gung Memorial Hospital, Taipei	1998 to 2002	1/78 (1.3%)	40.7% for contact lens wear 21.0% for ocular trauma	Hsiao et al., 2007
The Netherlands	All practicing ophthalmologists in the Netherlands	3-month period in 1996	1/92 (1.1%)	contact lenses	Cheng et al., 1999
New Zealand	Auckland Hospital	2 year period	1/98 (1.0%)	30% for previous ocular surgery 26% for contact lens wear 25% for topical corticosteroid use 24% for ocular trauma	Wong et al., 2003
South India	Aravind Eye Hospital and Postgraduate Institute of Ophthalmology, Tirunelveli, South India	September 1999 and August 2002	33/3183 (1.0%)	70.88% for ocular injuries 1.04% for contact lens usage 0.63% for usage of steroids 7.04% for systemic diseases	Bharathi et al., 2007

The Netherlands	Department of Ophthalmology of the Academic Medical Center (AMC) in Amsterdam or to the Rotterdam Eye Hospital (REH)	January 2002 and December 2004	0/156 (0%)	36.4% for systemic illness 33.8% for previous ocular surgery 28.6% for recurrent HED 26% for use of topical steroids 20.8% for blepharitis.	van der Meulen et al., 2008

[a] AK refers to *Acanthamoeba* keratitis and MK refers to microbial keratitis



Contact lens wearing is the most important risk factor of AK, associated with 100% AK cases in Australia (Ku et al., 2009), 96% AK cases in New Zealand (Patel et al., 2010) and 89% AK cases in United States (Verani et al., 2009) In the United States, a dramatic increase in AK cases paralleled with the growing popularity of soft contact lens during the 1980s (Stehr-Green et al., 1989), attributable to the use of non-sterile contact lens solutions, swimming with lenses, and inadequate disinfection on cleaning lenses (Stehr-Green et al., 1989). Contact lens wearers are 80-fold more likely to contract corneal infection than non-contact lenses users (Alvord et al., 1998; Dart et al., 1991). Indeed, AK cases have increased over the past twenty years (1987-2006) (Carvalho et al., 2009).

The association between wearing contact lenses and swimming was observed by Stehr-Green et al. (1989), indicating additional risk factor associated with *Acanthamoeba* keratitis is swimming or bathing in the non-disinfected aquatic environment while wearing contact lens. (Radford et al., 1998; Visvesvara, 1993).

The major risk factors of AK in developing countries differ from in developed countries, as no population use of contact lenses in developing countries. It has been identified even non-contact lens wearers are prone to AK (Sharma et al., 1990; Sharma et al., 2000; Srinivasan et al., 2003). Fall of dust particles, trauma due to vegetable matter, branch, sawdust in farmers, and contact with contaminated water, have been found to be predominant risk factors of AK (Kunimoto et al., 2000;

Lund et al., 1978; Radford et al., 2002; Tien and Sheu 1999). These factors are the major risk factors epically in farmers with AK. AK cases had been discovered in farmers worldwide (Ma et al. 1981; Manikandan et al., 2004; Srinivasan et al., 2003). The first case of GAE caused by *Acanthamoeba castellanii* in Taiwan occurs in a farmer (Liang et al., 2010; Sheng et al., 2009). There were five onion harvesters in the Heng-Chun Peninsula of southern Taiwan simultaneously reported suffering from fungal corneal ulcers, in which four out of five cases attributed to ocular trauma from pieces of onion skin or plant leaves drifted into their eyes while they were harvesting onions (Lin et al., 1999). Sun et al. (2006) discovered that, among 18 AK patients, six patients had a history of trauma with vegetative matter or dust, or had been recently exposed to swimming pool water. Manikandan et al. (2004) discovered half of 32 cases (16/32, 50.0%) were agricultural farmers and 8 (25.0%) of them were construction workers, injury by an object was the major predisposing factor (18, 54.2%). Ma et al. (1981) revealed the predisposing factor of two farmers with AK is trauma, one is caused by branch and another is caused by sawdust.

The first case of AK in Taiwan was reported in 1989 (Tseng et al., 1989). Table 1 indicates the studies on the prevalence of *Acanthamoeba*- induced microbial keratitis and the predominant risk factors in Taiwan. The incidence rate of microbial keratitis (MK) caused by *Acanthamoeba* in Taiwan varied from 1.3% to 13.9%; the predisposing factors are contact lens wearing and ocular trauma and only one case caused by overnight orthokeratology.

Overall, the above information suggests wearing contact lenses and cornea trauma followed by exposure to contaminated water, dust or particles are the important risk factors associated with AK.

1.2.3. Methods for characterization the potentially pathogenicity of *Acanthamoeba*

1.2.3.1. Genotyping

The classification of *Acanthamoeba* is continually under revision, following the successful application of molecular techniques (Booton et al., 2002; Booton et al., 2005; Stothard et al., 1998). The genus *Acanthamoeba* has been currently classified into 17 different genotypes based on rDNA sequence analyses, with a sequence divergence of 5% or more between different genotypes, i.e. T1–T12 (Stothard et al. 1998), T13 (Horn et al. 1999), T14 (Gast 2001), T15 (Hewett et al. 2003), T16 (Corsaro and Venditti, 2010), and T17 (Nuprasert et al., 2010). The most common genotype of *Acanthamoeba* worldwide is the genotype T4 (Booton et al., 2005), and up to 90% of isolates from AK patients belong to genotype T4 (Schroeder et al. 2001; Walochnik et al. 2000a, 2000b). Similarly, T4 has also been the genotype associated with GAE (Liang et al., 2010). However, genotype T1 (Fuerst et al., 2003), T2 (Maghsood et al., 2005; Walochnik et al., 2008), T3 (Booton et al., 2009; Ledee et al. 1996; Maghsood et al., 2005; Nagyova et al., 2010; Niyyati et al., 2010; Sharifi et al., 2010; Stothard et al. 1998; Zhang et al., 2004), T5 (Barete et al., 2007; Lackner et al.,

2010; Ledee et al., 2009; Spanakos et al., 2006), T6 (Walochnik et al., 2000b), T10 (Fuerst et al., 2003; Nuprasert et al., 2010), T11 (Khan et al., 2002; Lorenzo-Morales et al., 2011; Niyyati et al., 2009; Sharifi et al., 2010), T12 (Fuerst et al., 2003) and T15 (Di Cave et al., 2009; Nagyova et al., 2010; Sharifi et al., 2010) have known been isolated from the specimens of *Acanthamoeba*-infected patients and therefore been related with *Acanthamoeba* diseases. Comparisons between *Acanthamoeba* genotypes isolated from the environments and from AK and GAE patients can provide information on pathogenicity of environmental *Acanthamoeba*. Table 2 indicates the genotypes of clinical isolates.

Table 2 A current lists of genotypes and their association with the *Acanthamoeba* infections

Acanthamoeba genotypes	*Acanthamoeba* disease association	Reference
T1	GAE[a]	Fuerst et al., 2003
T2	AK[b]	Maghsood et al., 2005
	GAE	Walochnik et al., 2008
T3	AK	Sharifi et al., 2010; Niyyati et al., 2010; Nagyova et al., 2010; Booton et al., 2009; Maghsood et al., 2005; Zhang et al., 2004; Stothard et al. 1998; Ledee et al. 1996

T4	AK	Maubon et al., 2012; Prashanth et al., 2011; Zhao et al., 2010; Sharifi et al., 2010; Nagyova et al., 2010; Gatti et al., 2010; Chusattayanond et al., 2010; Abe and Kimata, 2010; Niyyati et al., 2009; Ledee et al., 2009; Di Cave et al., 2009; Booton et al., 2009; Yera et al., 2008; Ozkoc et al., 2008; Ertabaklar et al., 2007; Spanakos et al., 2006; Maghsood et al., 2005; Zhang et al., 2004; Sharma et al., 2004; Khan et al., 2002
	GAE	Liang et al., 2010
T5	AK	Ledee et al., 2009; Spanakos et al., 2006
	GAE	Lackner et al., 2010; Barete et al., 2007
T6	AK	Walochnik et al., 2000
T7	NA[c]	
T8	NA	
T9	NA	
T10	AK	Nuprasert et al., 2010
	GAE	Fuerst et al., 2003
T11	AK	Lorenzo-Morales et al., 2011; Sharifi et al., 2010; Niyyati et al., 2009; Khan et al., 2002
T12	GAE	Fuerst et al., 2003
T13	NA	
T14	NA	
T15	AK	Sharifi et al., 2010; Nagyova et al., 2010; Di Cave et al., 2009
T16	NA	

T17	NA

[a] GAE - Granulomatous Amebic Encephalitis

[b] AK - *Acanthamoeba* keratitis

[c] NA - no *Acanthamoeba* disease has been found

1.2.3.2. Thermotolerance testing

Once *Acanthamoeba* contact with human, they can tolerate body temperatures and growth with human body. Strains of *Acanthamoeba* that have the ability to grow or tolerate human body temperature (37°C and slightly higher) might infect humans, which is an important factor in pathogenicity (Khan and Tareen, 2003). Walochnik et al. (2000b) examined the thermotolerance of clinically relevant strains of *Acanthamoeba* and concluded that potentially pathogenic strains isolated from AK have high temperature tolerance (42^{o}C). Khan et al. (2001) tested nine isolates including pathogenic and nonpathogenic *Acanthamoeba*, and their results showed that all *Acanthamoeba* spp. grew at temperatures below 35°C, but only the pathogens grew at higher temperatures (37°C – 42°C). Table 3 summarizes the criteria of thermotolerance testing on the potential pathogenicity of *Acanthamoeba* isolated from environments. Three studies considered *Acanthamoeba* as pathogenic *Acanthamoeba* when they can grow at 37^{o}C (Khan and Tareen, 2003; Kilic et al., 2004; Lorenzo-Morales et al., 2006). Moreover, study was tested environmental *Acanthamoeba* at 37^{o}C and 42^{o}C and indicated that only pathogens can grow. Chan et al. (2011)

also tested the pathogenicity of *Acanthamoeba* grow at four different temperatures 37^{o}C, 42^{o}C. 46^{o}C and 52^{o}C (overnight prior to incubations at ambient temperature (26 ± 2^{o}C), and they considered *Acanthamoeba* that can survive and grow in human bodies which have an average body temperature of 37◦C, and are therefore considered as potential human pathogenic isolates.

Growth at temperature 37^{o}C and higher than 37^{o}C as the hallmark of pathogenic *Acanthamoeba*. The above studies show the ability of *Acanthamoeba* to grow at higher temperature (≥ 37^{o}C) correlated with the pathogenicity of *Acanthamoeba* isolates (Khan et al., 2001), and provide a good indicator on the pathogenic potential. Thus, we tested the response of *Acanthamoeba* isolated in present study at temperatures of 30^{o}C (as control), 37^{o}C, 42^{o}C and 52^{o}C.

Table 3 Criteria of thermotolerance testing on potential pathogenicity of *Acanthamoeba*

Culture condition	Temperature tested	Incubation period	Control group	Growth assessment	Reference
Each plate was centrally placed with a small NNA block saturated with cysts of *Acanthamoeba* isolates	37°C, 42°C, 46°C, and 52°C (overnight prior to incubation at ambient temperature (26±2°C)	7 days	A pathogenic strain of *A. castellanii* (ATCC 50492, originally from AK)	All plates were examined daily for migrating and proliferating trophozoites for 7 days.	Chan et al., 2011
A single trophozoite was transferred to a fresh NNA plate coated with heat-inactivated (1 h at 60°C) *E. coli*	37°C and 42°C	NA[a]	NA	Negative and positive for *Acanthamoeba*	Gianinazzi et al., 2010
Trophozoites were transferred (at 10^3 trophozoites/plate) to the centers of fresh plates of 1.5% NNA with overlayer of *E. coli* suspension	37°C and 42°C	10 days	*Acanthamoeba* trophozoites of each isolate was incubated at 30°C (as a control)	The number of trophozoites or cysts seen, about 20 mm from the center of each plate, in five microscope fields at x100 were counted, with counts of 0, 1 – 15, 16 – 30 and > 30 being scored as －, ＋, ＋＋ and ＋＋＋, respectively.	Caumo et al., 2009

Acanthamoeba isolates were inoculated on NNA overlaid with *K. aerogenes*	37^{o}C	NA	NA	Negative and positive for *Acanthamoeba*	Lorenzo-Morales et al., 2006
Acanthamoeba were inoculated onto NNA overlaid with *K. aerogenes*	37^{o}C	96 h	NA	Growth of organisms was determined by measuring the diameter of clearing zones in the bacterial lawn .	Kilic et al., 2004
Acanthamoeba isolates were inoculated on NNA overlaid with *K. aerogenes*	37^{o}C	72h	NA	By measuring the diameter of clearance in the bacterial lawn	Khan and Tareen, 2003

[a] Not available

1.2.3.3. Osmotolerance testing

The tear film, a layer of moisture which covers the eye, is the defense mechanism against outer objectives not only to protect the eyes but also maintain ocular moisture. *Acanthamoeba* are exposed to high osmolarity when they contact with tear film of human corneal. *Acanthamoeba* must withstand the osmotic stress and exhibit the growth for successful infection. The osmolarity of tear film has been reported to be averaged 304.4 ± 0.4 mOsmol/L with a range between 299 to 309 mOsmol/L (Gilbard and Rossi 1994; Gilbard 1994)

Once the pool isolates contact with human eyes, they can tolerant osmolarity of tear film and growth in human body. A correlation between osmotolerance (Khan 2001; Khan et al., 2002; Khan and Tareen, 2003) and pathogenicity of *Acanthamoeba* has been demonstrated. Khan et al. (2002) found that some pathogenic strains isolated from AK patients (Genbank assession number: AF239301, AF239303, AF239304, AF239305and AF239300) were all osmotolerant to 1 M mannitol in NNA, whereas1M mannitol equals to 0.25 osmolar, equal to 250 mOsmol/L (Khan and Tareen, 2003). The growth of non-pathogens (*A. palestinensis, A. polyphaga* CCAP 1501/3C, *A. comandoni* and *A. astronyxis*) was inhibited by 1 M mannitol. This suggests that pathogenic *Acanthamoeba* are able to grow at higher osmotic stress.

In terms of the methodology, Table 4 summarizes the criteria of osmotolerance testing on the potentially pathogenicity of *Acanthamoeba* isolated from environments. Khan and Tareen (2003)

discovered that pathogenic *A. polyphaga* ATCC30871 exhibit clearance in bacteria lawn of agar contain 1 M mannitol while non-pathogenic *A. polyphaga* CCAP 1501/3c was not able to grow. Kilic et al. (2004) determined that 12 out of 18 environmental isolates (66.6%) exhibited the growth at high osmolarity (1 M mannitol) and considered these isolates as potential pathogenic. Additionally, 5 out of 18 (27.7%) exhibited few trophozoites and limited growth and considered as weak potential pathogens, while one isolate (5.5%) exhibited no growth and was considered as non-pathogenesis. Lorenzo-Morales et al. (2006) and Chan et al. (2011) also tested the ability to growth at 1 M mannitol to differentiate the pathogenicity of environmental *Acanthamoeba*. Besides, Caumo et al. (2009) tested the ability to growth not only at 1 M mannitol but 0.5 M mannitol to differentiate pathogenicity of *Acanthamoeba* in environmental isolates.

Growth at osmolarity level 1 M mannitol is the hallmark of pathogenic *Acanthamoeba*. The above studies shown the ability of *Acanthamoeba* to grow at higher osmolarity (1 M mannitol) correlated with the pathogenicity of *Acanthamoeba* isolates (Khan 2003), and can be used as an indicator on the pathogenic potential of *Acanthamoeba*. Thus, we assessed the response of isolated *Acanthamoeba* at various osmolarity levels in non–nutrient agar (NNA) containing including 0 M mannitol (as control), 0.5 M mannitol, and 1 M mannitol.

Table 4 Criteria for osmotolerance testing on potentially pathogenicity of *Acanthamoeba*

Culture condition	Incubation temperature	Incubation period	Control group	Growth assessment	Reference
Small agar blocks containing *Acanthamoeba* cysts were placed centrally on NNA culture plates incorporated with 1M D-mannitol	26 ± 2^{o}C	7 days	A pathogenic strain of *A. castellanii* (ATCC 50492; originally from AK), was used as positive control.	All plates were examined daily for migrating and proliferating trophozoites for 7 days.	Chan et al., 2011
Trophozoites were transferred (at 10^3 trophozoites/plate) to the centers of fresh plates of 1.5% NNA containing no mannitol (as a control) or 0.5 or 1.0 M mannitol, each with the overlayer of *E. coli* suspension	30^{o}C	10 days	*Acanthamoeba* trophozoites of each isolate was incubated at 1.5% NNA containing no mannitol	Number of trophozoites or cysts seen, about 20 mm from the center of each plate, in five microscope fields at x100 were counted, with counts of 0, 1 – 15, 16 – 30 and > 30 being scored －, ＋, ＋＋ and ＋＋＋, respectively.	Caumo et al., 2009
Acanthamoeba isolates were inoculated on NNA overlaid with *K. aerogenes* and containing 1M mannitol	30^{o}C	NA[a]	NA	Negative and positive for *Acanthamoeba*	Lorenzo-Morales et al., 2006

Acanthamoeba were inoculated on NNA overlaid with *K. aerogenes* and containing 1 M mannitol	30^{o}C	96 h	NA	Growth of organisms was determined by measuring the diameter of clearing zones in the bacterial lawn	Kilic et al., 2004
Acanthamoeba isolates were inoculated on NNA overlaid with *K. aerogenes* and containing 1M mannitol	30^{o}C	72h	NA	By measuring the diameter of clearance in the bacterial lawn	Khan and Tareen, 2003

[a] Not available

1.2.3.4. Studies on potentially pathogenicity of *Acanthamoeba* isolated from the environments

Genotyping, thermotolerance and osmotolerance are the indicators in characterizing the potentially pathogenicity of *Acanthamoeba*. Characterizations the potentially pathogenicity of *Acanthamoeba* isolated from environments are by comparisons between genotypes of *Acanthamoeba* isolated from environments and with the genotypes of *Acanthamoeba* isolated from AK or GAE patients. To date, genotypes T1, T2, T3, T4, T5, T6, T10, T11, T12 and T15 had known been isolated from the specimens of *Acanthamoeba*-infectious patients. Thus *Acanthamoeba* isolates from environments are considered as potentially pathogenic if the genotype of environmental isolate belongs to T1 (Fuerst et al., 2003), T2 (Maghsood et al., 2005; Walochnik et al., 2008), T3 (Booton et al., 2009; Ledee et al. 1996; Maghsood et al., 2005; Nagyova et al., 2010; Niyyati et al., 2010; Sharifi et al., 2010; Stothard et al. 1998; Zhang et al., 2004), T4 (Liang et al., 2010; Schroeder et al. 2001; Walochnik et al. 2000a, 2000b), T5 (Barete et al., 2007; Lackner et al., 2010; Ledee et al., 2009; Spanakos et al., 2006), T6 (Walochnik et al., 2000), T10 (Fuerst et al., 2003; Nuprasert et al., 2010), T11 (Khan et al., 2002; Lorenzo-Morales et al., 2011; Niyyati et al., 2009; Sharifi et al., 2010), T12 (Fuerst et al., 2003) and T15 (Di Cave et al., 2009; Nagyova et al., 2010; Sharifi et al., 2010).

Growth at high temperature (usually higher than 37^{o}C) and high osmolarity (usually higher

than 1 M mannitol) are also the potentially pathogenic indicators for *Acanthamoeba*. Previous studies have shown that the ability of *Acanthamoeba* to grow at high temperature (37^{o}C) (Chan et al., 2011; Gianinazzi et al., 2010; Lorenzo-Morales et al., 2006; Kilic et al., 2004) and high osmolarity (1 M mannitol) correlated with the potentially pathogenicity of *Acanthamoeba* isolates (Chan et al., 2011; Caumo et al., 2009; Lorenzo-Morales et al., 2006; Kilic et al., 2004).

1.2.4. Environmental factors affecting *Acanthamoeba* presence in water

Rodriguez-Zaragoza (1994) indicated that the water parameters including pH, water temperature, and suspended particles that influence on the structure of amoebae communities and might affect their survival in aquatic environments, since amoebic resistance is dependent on cyst formation and trophozoite capacity for tolerating salinity, osmotic pressures. Another study also observed fecal coliform bacteria would contribute to the presence or concentrations of amoeba in aquatic habitats (Ettinger et al. 2003).

However, in terms of *Acanthamoeba* (Table 5) other previous studies indicated no apparent relationship between the presences of *Acanthamoeba* and two environmental factors, i.e. pH (Caumo et al., 2009; Huang and Hsu, 2010; Kao et al., 2011; Kawaguchi et al., 2009) and turbidity (Huang and Hsu, 2010; Kao et al., 2011; Kawaguchi et al., 2009). As for hardness, Radford et al. (2002) revealed that significant trend towards an increased incidence of AK with increasing

hardness of water supply. Behets et al. (2007) indicated *Acanthamoeba* were able to dominate when conductivity > 2000 μS/cm^2. There is no statistical correlation between water temperature and *Acanthamoeba* detection (Behets et al., 2007; Caumo et al., 2009; Huang and Hsu, 2010; Kawaguchi et al., 2009). However, Kao et al. (2011) revealed that an increase in water temperature was significantly with the presence of *Acanthamoeba* ($p=0.001$). In terms of disinfectant, Caumo et al. (2009) indicated no statistical significant ($P > 0.05$) between free chlorine concentration and presence or absence of *Acanthamoeba*. As for heterotrophic plate count (HPC), Kao et al. (2011) indicated no relationship significant between *Acanthamoeba* presence and HPC level, while Huang and Hsu. (2010) revealed the significant differences (Mann–Whitney U Test, $p < 0.05$) were observed in the HPC of samples that did and did not contain *Acanthamoeba*, but only when *Acanthamoeba* detected by combined two analytic methods were considered. Until now, no studies revealed the relationship between environmental factors and the potentially pathogenicity of *Acanthamoeba*.

Table 5 Studies on associations between environmental factors and presence of *Acanthamoeba* in aquatic environments

Environmental Factors	Sampling site	Results of tested environmental factors		Conclusion	Reference
		Mean	Range		
pH	Puzih and Kaoping River in southern Taiwan	APS[a]: 7.8:±0.34 ANS[b]: 7.9±0.36	APS:7.01–8.22 ANS: 6.65–8.76	No significant ($p = 0.087$).	Kao et al., 2011
	Tap water from houses in Mexico City	7.98±1.04	6.1–10.9		Bonilla-Lemus et al., 2010
	Spring, hot spring, and waste water in northern Taiwan	NA[c]	NA	No significant differences.	Huang and Hsu, 2010
	Heated and unheated swimming pools in Brazilian state.	NA	Heated swimming pools: 7.2–8.2 Unheated pools: 7.0–8.2	No significant ($P > 0.05$)	Caumo et al., 2009
	River water from public parks in Sapporo City	7.22 ± 0.75	NA	No statistical correlation.	Kawaguchi et al., 2009

Turbidity (NTU)	Puzih and Kaoping River in southern Taiwan	APS: 117.9±86.79 ANS: 124.8±377.08	APS: 10–289 ANS: 11–5,000	No significant ($p = 0.665$).	Kao et al., 2011
	Spring, hot spring, and waste water in northern Taiwan	NA	NA	No significant differences.	Huang and Hsu, 2010
	River water from public parks in Sapporo City	5.28 ± 6.28	NA	No statistical correlation.	Kawaguchi et al., 2009
Hardness (mg/L as $CaCO_3$)	No sampling (Epi study)	NA	NA	Significant trend towards an increased incidence of *Acanthamoeba* keratitis (AK) with increasing hardness of water supply	Radford et al., 2002
Conductivity (μS/cm)	Tap water from houses in Mexico City	499.16±241.06	159–866		Bonilla-Lemus et al., 2010

	Water in Belgian electrical power plants	The mean conductivity of the 108 other samples was 824±271	Conductivity of all 15 Rdh samples ranged from 3,820 to 9,350	Conductivity > 2000 $\mu S/cm^2$, *Acanthamoeba* are able to dominate.	Behets et al., 2007
Water temperature (^oC)	Puzih and Kaoping River in southern Taiwan	APS: 29.2±3.56 ANS: 25.3±4.46	APS: 20.3–32.5 ANS: 18.4–33.2	Significantly with absence of *Acanthamoeba* ($p = 0.001$).	Kao et al., 2011
	Tap water from houses in Mexico City	22.44±3.63	16–34		Bonilla-Lemus et al., 2010
	Spring, hot spring, and waste water in northern Taiwan	NA	NA	No significant differences.	Huang and Hsu, 2010
	River water from public parks in Sapporo City	20.31 ± 4.27	NA	No statistical correlation.	Kawaguchi et al., 2009
	Heated and unheated swimming pools in Brazilian state.	NA	Heated swimming pools: 23–35 Unheated pools: 14–26	No significant affect ($P > 0.05$).	Caumo et al., 2009

	Water in Belgian electrical power plants	24.99±3.84	17.3-35.2	No correlation was revealed.	Behets et al., 2007
Dissolved Oxygen (mg/L)	Tap water from houses in Mexico City	3.32±0.72	2.0–4.8		Bonilla-Lemus et al., 2010
Free chlorine concentration (mg/L)	Tap water from houses in Mexico City	0.24±0.33	0.0–1.14		Bonilla-Lemus et al., 2010
	Heated and unheated swimming pools in Brazilian state.	NA	Heated swimming pools: 1.0–4.0 Unheated pools: 1.0–4.0	No significant ($P > 0.05$)	Caumo et al., 2009
Heterotrophic plate count, HPC (CFU/mL)	Puzih and Kaoping River in southern Taiwan	APS: $3.1\times10^4\pm3.98\times10^4$ ANS:$3.7\times10^4\pm1.08\times10^5$	APS: $885–2.05\times10^5$ ANS: $50–1.09\times10^6$	No significant ($p = 0.071$)	Kao et al., 2011
	Spring, hot spring, and waste water in northern Taiwan	NA	NA	Significant differences were observed between the *Acanthamoeba*-positive and the negative	Huang and Hsu, 2010

	samples for HPC (p = 0.049935), but only when combined PCR and culture methods

[a] APS: *Acanthamoeba*-positive samples.

[b] ANS: *Acanthamoeba*-negative samples.

[C] Not available

1.2.5. Environmental factors affecting *Acanthamoeba* in soil

The occurrence of *Acanthamoeba* in soil may be contributed to the cysts form of *Acanthamoeba*, which was important for the presence and survival of *Acanthamoeba* in stressful condition. There are no studies on associations between environmental factors and presence of *Acanthamoeba* in soil. For this reason it is of interest to study *Acanthamoeba* in the soil, along with the environmental conditions favoring their survival, so as to formulate more effective control measures. Until now, the relationships between the environmental factors and the abundance of *Acanthamoeba* are still inconsistent and unclear.

1.2.6. Environmental factors affecting *Acanthamoeba* in air

The occurrence of *Acanthamoeba* in air may be contributed to the cysts form of *Acanthamoeba*, which was important for the presence and survival of *Acanthamoeba* in stressful condition. Table 6 illustrates the studies on associations between environmental factors and presence of amoeba or *Acanthamoeba* in air. Rivera et al. (1994) mentioned that the main source of air-borne amoebae was from the soil, and the factors favored the incidence and diversity of amoebae were wind speed and wind direction, low relative humidity, generation of frequent dust-storms, re-suspension of amoebae by vehicular traffic, and large extensions of bare soil. Rodriguez-Zaragoza and Magana-Becerra. (1997) indicated that the isolations of *Acanthamoeba* were more abundant during the dry season and

the sanitary conditions may enhance the proportion of pathogenic strains in the surroundings. For this reason it is of interest to study which amoeba species or other protozoa are scattered by the air, along with the environmental conditions favoring their dispersal, so as to formulate more effective control measures. Until now, the relationships between the environmental factors and the abundance of *Acanthamoeba* are still inconsistent and unclear.

Table 6 Studies on associations between environmental factors and presence of amoeba or *Acanthamoeba* in air

Environmental Factors	Sampling site	Conclusions	Reference
Soil	South Dakota School of Mines and Technology campus, Rapid City	All the protozoa sampled were typical soil forms suggesting that most of the particles sampled in this study were derived from soils	Rogerson and Detwiler, 1999
	Mexico City and its metropolitan area	The main source of air-borne amoebae was the soil	Rivera et al., 1994
Wind	South Dakota School of Mines and Technology campus, Rapid City	Generally, more soil particles and protozoa are in the air on windier days	Rogerson and Detwiler, 1999
Wind velocity	Mexico City and its metropolitan area	Significant correlations were found between wind velocity ($r = 0.79$; $p < 0.05$) and number of isolated amoebae	Rivera et al., 1994
Rain	South Dakota School of Mines and Technology campus, Rapid City	Rainfall can have a strong influence since rain washes protozoan cysts out of the air and ground moisture prevents their resuspension into the air by wind currents	Rogerson and Detwiler, 1999
	City of San Luis Potosi, Mexico	More than 60% of the *Acanthamoeba* were isolated during the hottest and driest months (April to May)	Rodriguez-Zaragoza and Magana-Becerra, 1997

Relative Humidity	City of San Luis Potosi, Mexico	More than 60% of the *Acanthamoeba* were isolated during the hottest and driest months (April to May).	Rodriguez-Zaragoza and Magana-Becerra, 1997
	Mexico City and its metropolitan area	The highest presence of *Acanthamoeba* during the months of less relative humidity may be explained due to the cellulose content of the cyst wall that make these amoebae more resistant to dehydration	Rivera et al., 1994
Temperature	City of San Luis Potosi, Mexico	More than 60% of the *Acanthamoeba* were isolated during the hottest and driest months (April to May.	Rodriguez-Zaragoza and Magana-Becerra, 1997
	Mexico City and its metropolitan area	Non-significant correlations were found between wind velocity ($r = -0.03$; $P > 0.05$) and number of isolated amoebae	Rivera et al., 1994
Dust-storms	Mexico City and its metropolitan area	This station was the most exposed to air currents which generate dust-storms that travel from the north and northeast of the city to the south	Rivera et al., 1994
Vehicles	Mexico City and its metropolitan area	Heavy vehicular traffic in the area which may cause the resuspension of microorganisms	Rivera et al., 1994

Trash dump	City of San Luis Potosi, Mexico	The higher incidence was found in the suburban and urban zones because they share common sanitary conditions such as waste disposed directly on the streets	Rodriguez-Zaragoza and Magana-Becerra, 1997
	Mexico City and its metropolitan area	This station lies in close proximity to an open-air trash dump and to large areas of bare soil both of which may provide a source of amoebae	Rivera et al., 1994
Large areas of bare soil	City of San Luis Potosi, Mexico	The higher incidence was found in the suburban and urban zones because they share common sanitary conditions such as lack of pavement	Rodriguez-Zaragoza and Magana-Becerra, 1997
	Mexico City and its metropolitan area	This station lies in close proximity to an open-air trash dump and to large areas of bare soil both of which may provide a source of amoebae	Rivera et al., 1994

1.3. Rationales of this study

Swimming pools are one of the recreational water environments that people often contact with in their daily life activities. AK occurs through wearing contact lenses while bathing or swimming in contaminated aquatic environment (Khan and Tareen, 2003; Marciano-Cabral and Cabral, 2003; Martínez and Visvesvara, 1997; Parija et al., 2001; Radford et al., 1998; Schuster and Visvesvara, 2004; Sharma et al., 2000). The risk of AK is greater when wearing contact lenses while swimming (Stehr- Green et al., 1987). Besides, it was reported that *Acanthamoeba* being the most prevalent species in swimming pool waters, and a relatively high percentage of the amoebae in swimming pool water is pathogenic compared to the population in other waters (Khan 2006). The study from De Jonckheere and Van de Voorde (1976) discovered pathogenic *Acanthamoeba* strains were much more resistant to chlorine compared to those nonpathogenic.

Onion farmlands located in Hengchun and Checheng Townships of Pingtung County, where generated autumn tempest from October to April. Wind velocity of the autumn tempest ranging from 10 to 17 m/s, acting just like tropical storm or seldom typhoon. The autumn tempest not only generates bioaerosols transported in the air easily but brings the particulate, vegetative matter or dust that can result in trauma on the eyes. Onion harvesters in the HengChun Peninsula of southern Taiwan simultaneously reported suffering from fungal corneal ulcers may attribute to ocular trauma caused by pieces of onion skin or plant leaves that drifted into their eyes while they were harvesting

onions (Lin et al., 1999). Trauma has been known to the major risk factors especially in farmers with AK (Ma et al., 1981; Manikandan et al., 2004; Sun et al., 2006).

Acanthamoebidae may be more favored for aerial dispersal due to the cyst formation. The field survey on pathogenicity of *Acanthamoeba* in farmlands is important, since the autumn tempest can not only generate bioaerosols transported in the air but bring the particulate, vegetative matter or dusts that can result in trauma on the eyes, which could present a lethal threaten to farmers' health. Besides, previous studies indicated the predisposing factors of AK are contact lens wearers and ocular trauma in Taiwan (Chen et al., 2004; Fong et al., 2004; Hsiao et al., 2007).

There is no information for the potentially pathogenicity of *Acanthamoeba* in swimming pools and onion farmlands of Taiwan and thus swimming pools and onion farmlands are the environments undertaken to assess the potential of *Acanthamoeba* infections. Therefore, this study was carried out to detect potentially pathogenicity of *Acanthamoeba* by genotype, thermotolerance and osmotolerance from swimming pools and onion farmlands in order to provide public health information in Taiwan.

Chapter 2 Objectives of the Study

2.1. Identify *Acanthamoeba* species and genotypes isolated from chlorinated swimming pools in Taipei City and onion farmlands in Pingtung County.

2.2. Characterize the potentially pathogenicity of *Acanthamoeba* based on genotypes, thermotolerance and osmotolerance.

2.3. Assess the influence of environmental factors on the distribution of potentially pathogenic *Acanthamoeba*.

Chapter 3 Framework of the study

To accomplish the aims of the present study, identify *Acanthamoeba* species and genotypes isolated from chlorinated swimming pools and onion farmlands with characterizations of the potentially pathogenicity on *Acanthamoeba* isolates through genotypes, thermotolerance and osmotolerance. Further assess on the influence of environmental factors on the distribution of pathogenic *Acanthamoeba*. The study skeleton is shown as below.

3.1. Sampling from swimming pools and onion farmlands

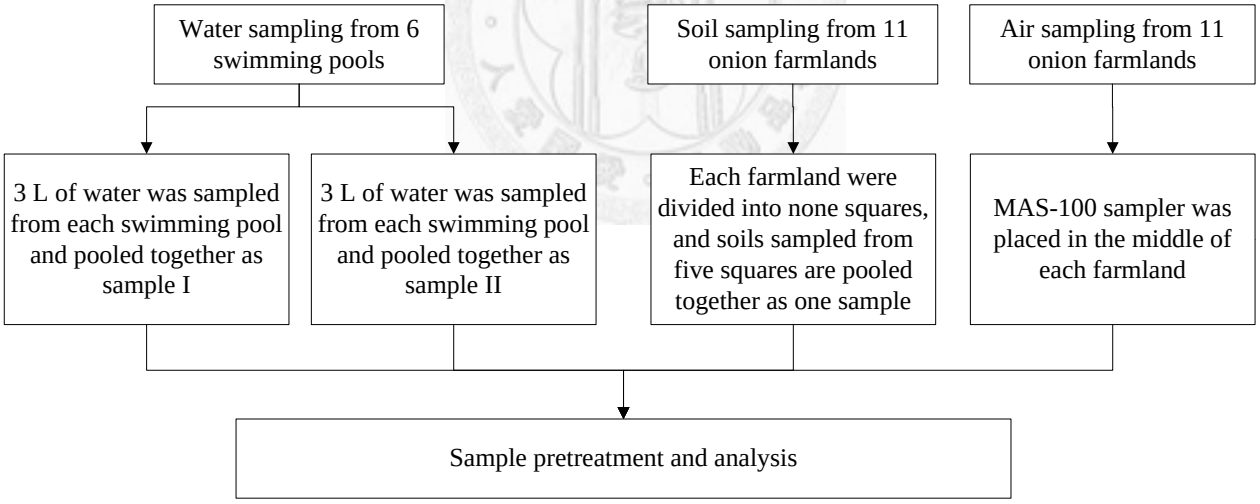


Figure 1 Sampling from swimming pools and onion farmlands

3.2. Identification and characterization of *Acanthamoeba* from

swimming pools and onion farmlands

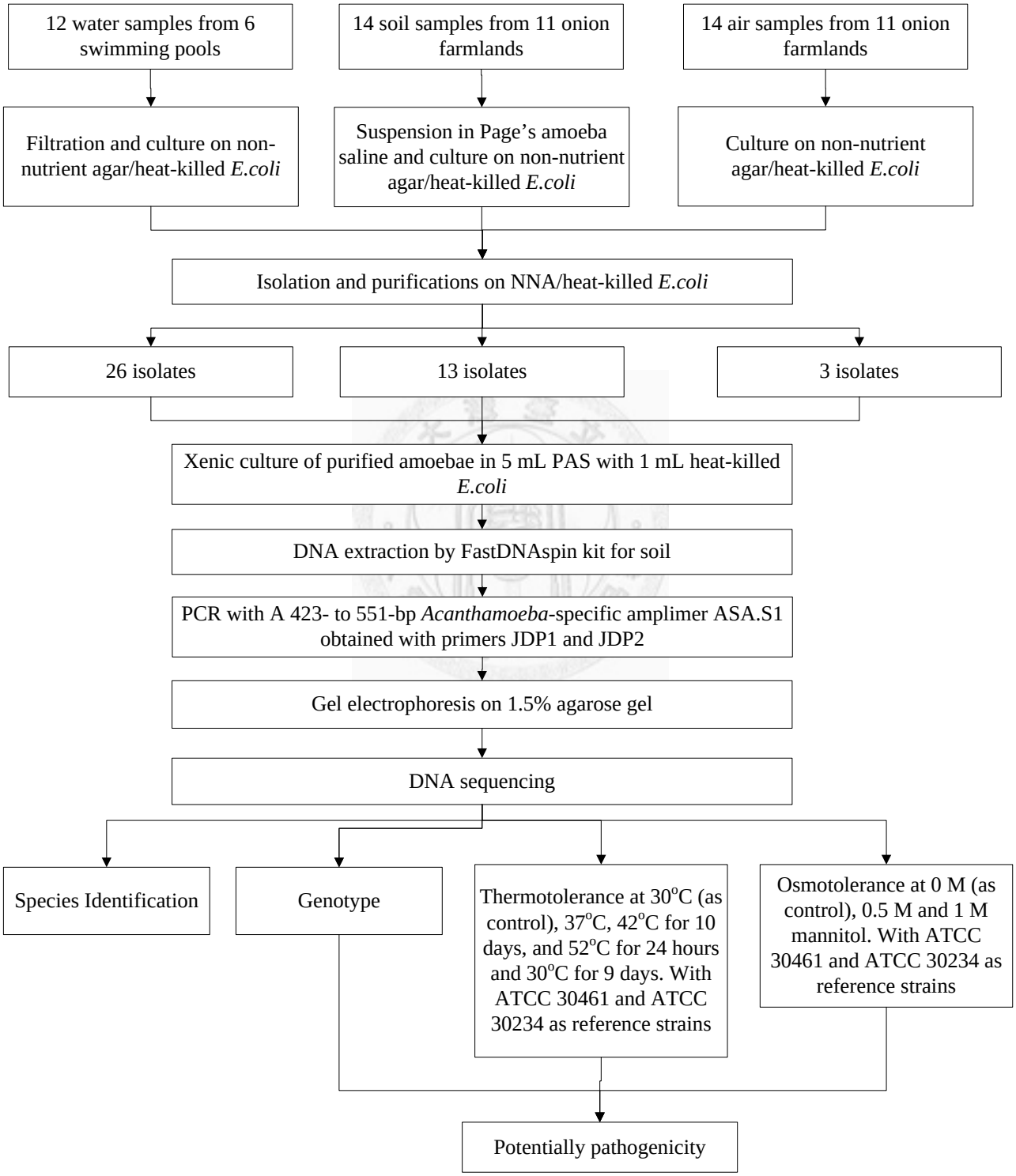


Figure 2 Identification and characterization of *Acanthamoeba* pathogenicity from swimming pools and onion farmlands

3.3. Assessment the influence of environmental factors on the distribution of potentially pathogenic *Acanthamoeba*

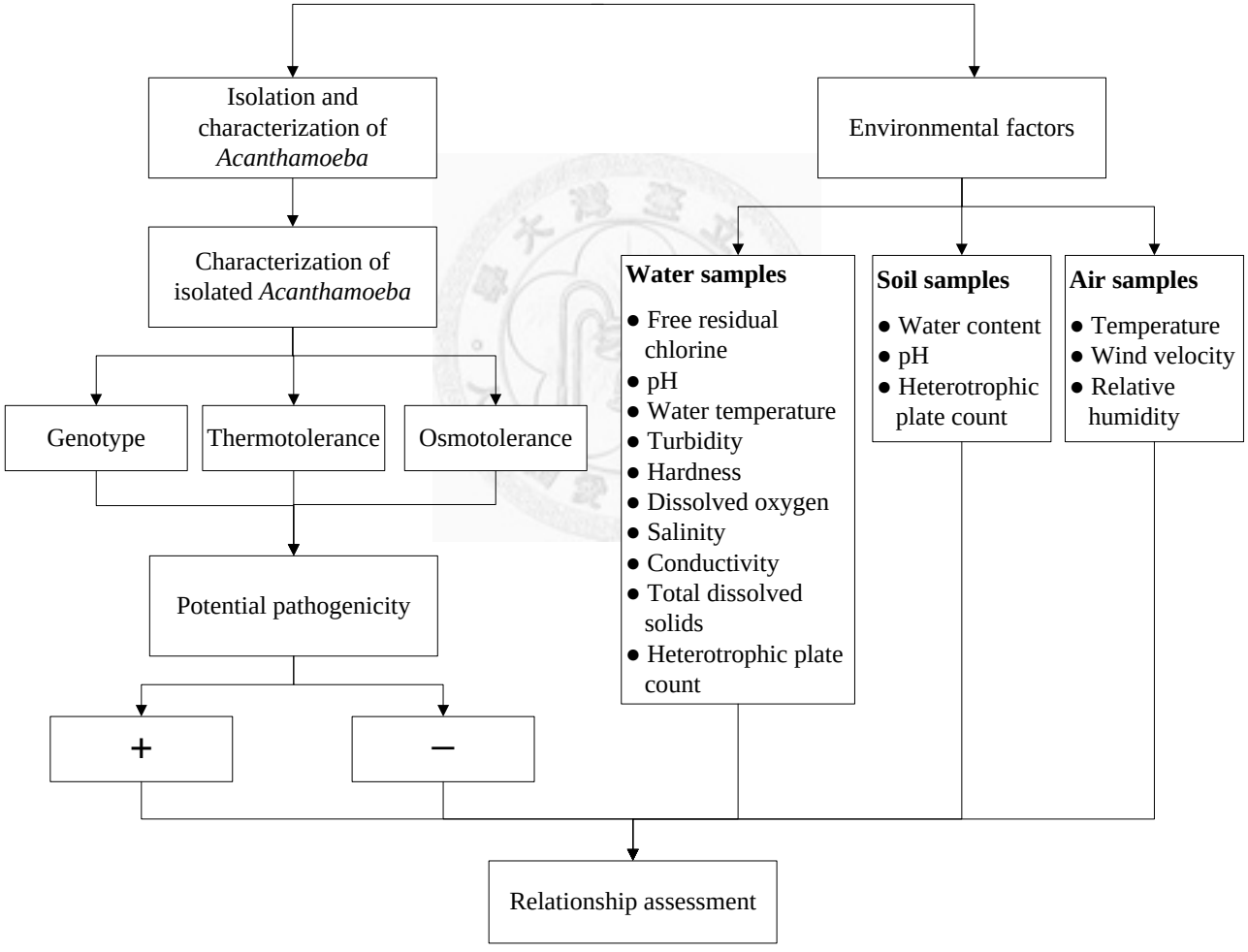


Figure 3 Assessment the influence of environmental factors on the distribution of *Acanthamoeba* potentially pathogenicity

Chapter 4 Material and Methods

4.1. Culture medium and buffer solution

4.1.1 Preparation of Page's Amoeba Saline (PAS)

Table 7 Composition of PAS (Page, 1988)

Chemical reagents	Concentration	Quantity
NaCl	0.2 M	1.20 g
$MgSO_4 \cdot 7H_2O$	0.001 M	0.04 g
$CaCl_2$	0.0036 M	0.04 g
Na_2HPO_4	0.0568 M	1.42 g
KH_2PO_4	0.999 M	1.36 g

The chemical reagents are shown in Table 7. NaCl (Sigma-Aldrich (31434), USA), $MgSO_4 \cdot 7H_2O$ (J.T.Baker (2500-01), USA), $CaCl_2$ (Sigma-Aldrich (C3306), USA), Na_2HPO_4 (Sigma-Aldrich (30427), USA), KH_2PO_4 (J.T.Baker (3246-01), USA) were separately added into 100 mL deionized water to prepare the stock solutions. Afterwards, 10 ml of each stock solution were mixed with 950 ml of deionized water to make the total volume of 1 L, which was stored at room temperature after autoclaved at 121°C for 20 minutes.

4.1.2. Preparation of ATCC medium 712

Table 8 Composition of ATCC medium 712

Chemical reagents	Concentration	Quantity or Volume

Proteose peptone		20.0 g
Yeast extract		1.0 g
$MgSO_4$	0.4 M	10.0 mL
$CaCl_2$	0.05 M	8.0 mL
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	0.005 M	10.0 mL
$Na_2HPO_4 \cdot 7H_2O$	0.25 M	10.0 mL
KH_2PO_4	0.25 M	10.0 mL
Sodium citrate	0.1 M	34.0 mL
Glucose	2M	50.0 mL

Proteose peptone (20 g) and Yeast extract (1g) were well-mixed with 950 ml deionized water and autoclaved at 120 ^{o}C for 20 minutes with $MgSO_4$ (J.T.Baker (2506), USA), $CaCl_2$ (Sigma-Aldrich (C3306), USA), $Fe(NH4)_2(SO4)_2 \cdot 6H_2O$ (Sigma-Aldrich (203505), USA), $Na_2HPO_4 \cdot 7H_2O$ (Sigma-Aldrich (30427), USA), KH_2PO_4 (J.T.Baker (3246-01), USA), Sodium citrate (3646-01), USA), glucose (Sigma-Aldrich (G7021), USA), proteose peptone (Sigma-Aldrich (P0431), USA) and yeast extract (BD Bacto (212750), USA) were prepared separately and sterilization at 121°C for 20 minutes. After the sterilization, the salts were mixed with the 950 ml solution containing Proteose peptone and Yeast extract. The 2 M Glucose were well mixed into the medium through the 0.22-μm pore size aseptic filtration (Millipore, Bedford, MA) and dispensed to the 50ml sterile centrifuge tubes. The tubes were placed at room temperature overnight and checked for sterilization. The ATCC medium 712 was stored at 4^{o}C.

4.1.3. Nutrient Agar (NA)

The 23 g of NA powder (BD Bacto (213000), USA) was suspended in 1 L of deionized water and mixed thoroughly. After autoclaved at 121^{o}C for 20 minutes, the agar was dispersed into 90 mm petri dishes and stored at 4^{o}C.

4.1.4. R2A agar

The 18.2 g of R2A Agar powder (Difco™ R2A Agar (299436), BD, USA) was suspended in 1 L of deionized water and mixed thoroughly. After autoclaved at 121°C for 20 minutes, the agar were dispersed into 90 mm petri dishes and stored at 4^{o}C.

4.1.5. Non-Nutrient agar (NNA)

The 15 g of bacteriological agar (Difco™ R2A Agar (214010), BD, USA) was suspended in 1 L of PAS and mixed thoroughly. After autoclaved at 121°C for 20 minutes, the agar were dispersed into 90 mm petri dishes and stored at 4^{o}C.

4.1.6. Preparation Non-Nutrient agar containing 0.5 M mannitol and 1M mannitol

Non-nutrient agar containing 0.5 M mannitol were made with 15 g of bacteriological agar and

91.085 g D-mannitol (Sigma-Aldrich (M4125), USA) were thoroughly dispersed in 1 L of PAS, then sterilization by autoclaving at 121^{o}C for 15 minutes, and dispersed into 90 mm petri dishes and stored at 4^{o}C. Non-nutrient agar containing 1 M mannitol were made with 15 g bacteriological agar and 182.17 g D-mannitol were thoroughly dispersed in 1 L of PAS, then sterilization by autoclaving at 121^{o}C for 15 minutes, and dispersed into 90 mm petri dishes and stored at 4^{o}C.

4.1.7. NNA plates seeded with heat-killed *E.coli* (heat-killed *E.coli*/NNA plate)

All *E.coli* colonies on NA were recovered with a sterile swab, suspended in 2 mL of sterile PAS and heated at 70°C for 2 hours (Lanocha et al., 2009). Using a sterile plastic Pasteur pipette, three drops of heated *E. coli* suspension was inoculated on to the center of a NNA plate. *E. coli* were spread evenly over the surface of the agar using a sterile hockey stick or swab.

4.1.8. Preparation of 1X Tris-acetate-EDTA (TAE) buffer

The 20 mL of 50X TAE buffer (UltraPure™ (24710-030) USA) was diluted with 980 mL deionized water, and the bottle of 1X buffer solution was stored at room temperature.

4.1.9. Preparation of 1.5 % gel

For 1.5 % gel, 0.75 g agarose (SeaKem® LE Agarose) was adding to 50 mL 1x TAE buffer,

and the solution was heated to boiling in the microwave to dissolve the agarose. The two dams were put into the slots on each side of the gel plate. The gel was then cooled to temperature that could hold the bottle with bare hands before pouring the melted agarose onto the gel plate in the eletrophoresis box. The dams and the comb were removed carefully when the gel was solid.

4.2. Microbial strains and preparation

Acanthamoeba polyphaga (ATCC30461), obtained from the American Type Culture Collection, was originally isolated from human corneal scrapings of a case of AK (Visvesvara et al., 1975) and therefore identified as potentially pathogenic strain in the present study. *A. polyphaga* (ATCC30461) were transferred in ATCC medium 712 and incubated at 25^{o}C for 7 days and repeated this procedure every week.

A. castellanii (ATCC30234) was obtained from the ATCC, originally from yeast culture, United Kingdom (Visvesvara and Balamuth, 1975). The cytotoxicity determined using lactate dehydrogenase, LDH assays revealed 51.5 ± 4.5 reported by Maghsood et al. in 2005, and thus recognized *A. castellanii* (ATCC30234) as probably pathogenic strain. *A. castellanii* (ATCC30234) were transferred in ATCC medium 712 and incubated at 25^{o}C for 7 days and repeated this procedure every week.

E.coli (ATCC25922) was obtained from the ATCC, originally from clinical isolate (Boyle et al.,

1973). A single colony of a routine laboratory strain of *E. coli* NCTC 9001 was spread onto a nutrient agar plate and incubates overnight at 37°C. Store in refrigerator and re-new each week.

4.3. Quality assurance and quality control on thermotolerance and osmotolerance pretesting

4.3.1. Preparation of *Acanthamoeba* samples

The assay was conducted to evaluate the distance of *Acanthamoeba* migrating during proliferation at various temperatures and different osmolarity level. *A. polyphaga* (ATCC 30461) and *A.castellanii* (ATCC30234) were transferred in ATCC medium 712 and incubated at 25^{o}C for three days to form trophozoites. Cell suspensions were moved to sterile centrifugation tubes (15 mL) by an aseptic autopipette, and centrifuged at 200 x g for 8 minutes at 4^{o}C to collect the cells (Grimm et al., 2001). Cell pellets were then re-suspended in 1 mL sterilize PAS and the amoebic concentration were determined by a hemocytometer (Marienfeld, Lauda-Konigshofen, Germany). Approximately 10 μL of *Acanthamoeba* was carefully transferred to one of the semi-reflective panels on a hemocytometer covered with a cover slip. There was a grid of 9 squares in upside and 9 squares in downside under the microscope. There were 16 smaller squares in a square and the numbers of *Acanthamoeba* were counted in each smaller square. Following equation was used to calculate the number of cells in the original volume: (Total cells/Squares counted) *10^4*dilution

factor = Cells/mL in original suspension. With appropriate volume, cell suspension was serially diluted with sterile PAS to make a final cell suspensions at a concentration of 10^5 trophozoites in 1 mL PAS, which was used for thermotolerance and osmotolerance experiments.

4.3.2. Distance of *Acanthamoeba* ATCC strains migrated after two hours inoculum onto NNA/heat-killed *E.coli*

To evaluate the distance of *A. polyphaga* (ATCC 30461) and *A.castellanii* (ATCC 30234) were attribute to *Acanthamoeba* migration ability, not the initial inoculum. The test was started with initial inoculum of *A. polyphaga* (ATCC 30461) and *A.castellanii* (ATCC 30234) on 0 M and 1 M NNA/heat-killed *E.coli* and positioned for two hours in a biosafety cabinet, during this time *Acanthamoeba* were settled down to surface of agar. The *Acanthamoeba* ATCC trains were not shifted on NNA plates when plates were moved to invert microscopic for counting the distance of *Acanthamoeba* ATCC trains at the initial inoculum. The results were quantitatively through enumeration *Acanthamoeba* under inverted microscopic at 100X at each microscopic field. Migration of *Acanthamoeba* in each testing were occurred once the the *Acanthamoeba* trophozoite and cyst presence at area out of first inoculation,

4.3.3. Thermotolerance testing

An aliquot of 10 µL of 10^3 trophozoites was transferred to the center of fresh 1.5% NNA plates pre-covered with heat-killed *E. coli* suspension, sealed with Parafilm and positioned for two hours in a biosafety cabinet. The plates were then incubated at 30°C (as control) (Caumo et al., 2009), 37°C (Caumo et al., 2009). *Acanthamoeba* isolates can survive and grow in human bodies which have an average body temperature of ±37◦C, and are therefore considered as potential human pathogenic isolates. And 42°C for 10 days (Chan et al., 2011) in order to investigate *Acanthamoeba* for increased thermotolerance. To investigate the effects of higher temperature on viability of *Acanthamoeba,* cysts were exposed 52°C (Chan et al., 2011) for 24 hours prior to incubations at 30°C for nine days, which favored excystation. Each of *Acanthamoeba* strains was tested in triplicate at a given temperature.

4.3.4. Osmotolerance testing

An aliquot of 10 µL of 10^3 trophozoites were transferred to the center of fresh 1.5% NNA plates containing no mannitol (as control) and 1 M mannitol. Each plate was pre-covered with heat-killed *E. coli* suspension. After cell inoculation, the plates were sealed with parafilm and positioned for two hours in a biosafety cabinet, followed by incubation at 30°C for 10 days (Caumo et al., 2009). Each of *Acanthamoeba* strain was tested at 0 M and 1M mannitol in triplicate.

4.3.5. Evaluation of the level of *Acanthamoeba* growth for thermotolerance and osmotolerance testing

The number of trophozoites and cysts of *A. polyphaga* (ATCC 30461) and *A.castellanii* (ATCC 30234) were counted daily under inverted microscopic (Eclipse TE2000-U, Nikon, Tokyo, Japan) at 100X in every microscope fields from the center of each plate, as shown in (Fig. 4). The distance away from the plate center was divided into two sections, section I was 15 to 25 mm away from the center of NNA plate and section II was 26 to 35 mm away from the center of the plates. The number of *Acanthamoeba* cells (trophozoite and cyst) in microscope fields of at section I and II were averaged, respectively, for triplicate plates on every day. The results of evaluations on *Acanthamoeba* were determined by the maximum number of *Acanthamoeba* trophozoite and cyst on which incubation day and the distance away from the center of NNA. *Acanthamoeba* cyst presence out of the first inoculation area means *Acanthamoeba* had been migration to the area. *Acanthamoeba* trophozoites presence out of the first inoculation area means *Acanthamoeba* are migrating to the area. Therefore, trophozoite and cyst were counted for evaluated the growth of *Acanthamoeba.*

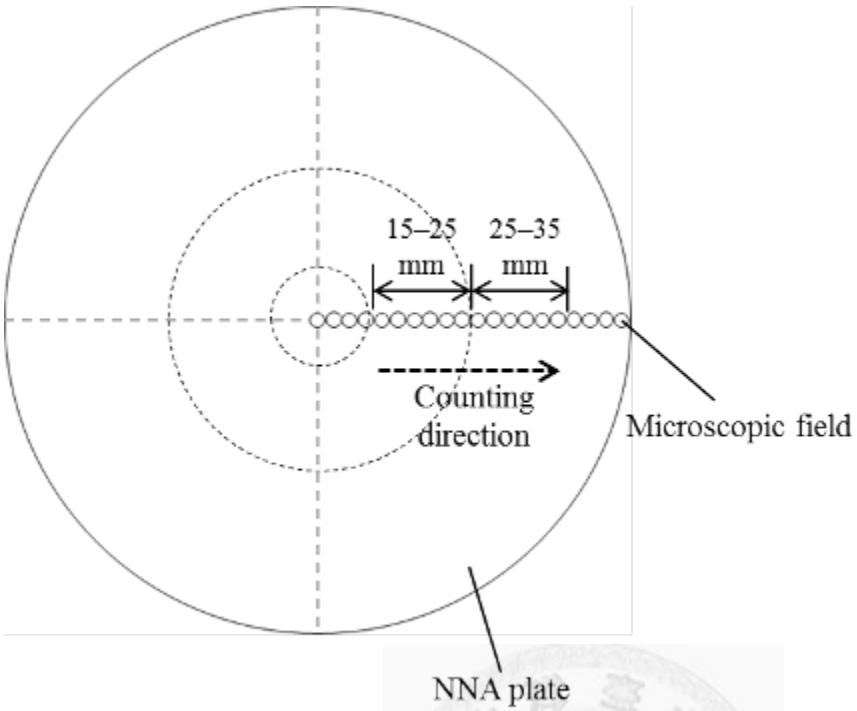


Figure 4 Illustration of evaluations on thermotolerance and osmotolerance

4.4. Sampling on swimming pools and onion farmlands

4.4.1. Water samples from swimming pools

Water samples were collected from six swimming pools during normal operation in Taipei, Taiwan (July 2 and July 16) of 2011 and (early spring March 21 and early spring March 28) of 2012. Four swimming pools were located at community-type swimming pools in Taipei City (Swimming pool A, B, C and D), while the other two swimming pools were located at the school-type in Taipei City (E and F). Sampling time of four community-type swimming pools were in the morning around 09:00 a.m. to 10:00 a.m. Sampling time of two school-type swimming pools were in the

afternoon around 02:00 p.m. to 03:00 p.m. Five out of six swimming pools were automatic filtration followed by automatic chlorination for disinfections. One school-type, swimming pool E was used automatic filtration followed by human adding chlorine for disinfections. Total six L of pool water (i.e. six of one L water) was taken from each swimming pool on each sampling. In detail, one L of water was collected from six different positions in a swimming pool on one side (Fig. 5) or two sides (Fig. 6) of the pool, in order to obtain representative samples.

Pool water (one L) was taken under the water surface 15 cm of swimming pool (Chang et al., 2010) at the position at least six m away from the effluent exit and from the influent exit, and one m away from the edge of the swimming pool (Leoni et al., 2001; Rabi et al., 2007). The distance between water samples was three meters. The pool water (one L) was stored in a sterile fluorinated wide mouth bottles (Nalgene®) containing 1 mL of sterile $Na_2S_2O_3$ (10 %) (Leoni et al., 1999; Leoni et al., 2001). Three of one L pool water sample were then pooled together as sample I, and the other three of one L of pool water were also pooled together as sample II (Fig. 5 and 6). Thus, there were 12 water samples (three L each from six pools). Water samples were transported under refrigeration (Ettinger et al., 2003) to the laboratory within two hours and processed less than four hours.

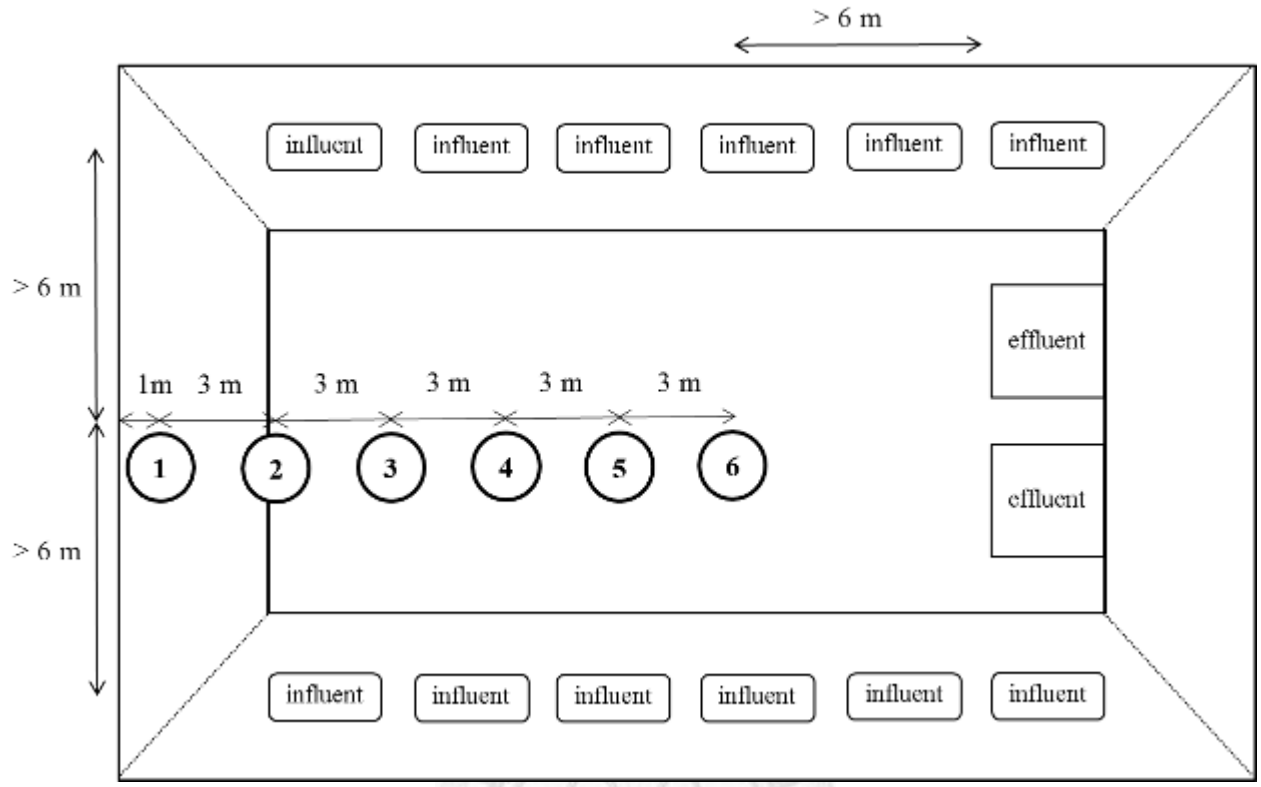


Figure 5 The overlook of one-side sampling in swimming pool. Circle with number represents the plates where water sample (one L each) taken. Samples 1 – 3 were combined as sample I, whereas sample 4 – 6 were combined as sample II.

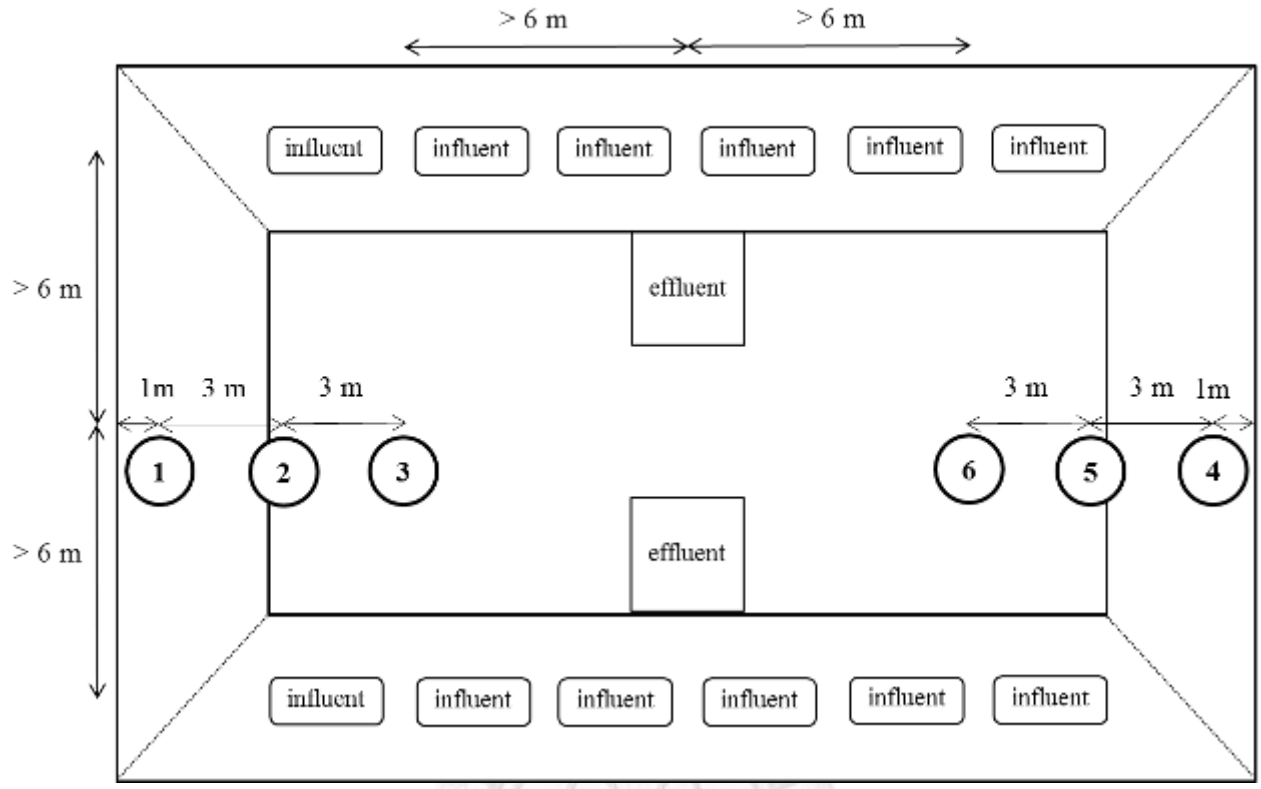


Figure 6 The overlook of two-sides sampling in swimming pool. Circle with number represents the plates where water sample (one L each) taken. Three samples collected on the same side were combined as sample I for 1 – 3 and sample II for 4 – 6.

4.4.2. Soil and air samples from farmland

Soil and air sampling were conducted by Mr. Chung-Long Kuo during early winter (November 20 – November 23) of 2011 and early spring (March 19 – March 24) of 2012. Totally, 14 soil and 14 air samples were collected from 6 onion farmlands of Hengchun Township and five onion farmlands in Checheng Township, Pingtung County, Taiwan (Fig. 7). Sampled onion farmlands

were located away from the road (at least one farmland away from the road), the distance between sampled farmlands were at least 100 m. For each selected farmland, it was divided into nine equal area and soils were collected by shovel at sampling area of 10 cm^2 and a sampling depth of one cm and from the center of five area, i.e. left-top, right-top, left-down, right-down, and middle squares (Fig. 8a). Soils collected from five squares were combined into one sample, placed into a zip lock bag, well-mixed, and transported under refrigeration to the laboratory within two days.

Air sampling was conducted at the center of each onion farmland in order to represented each onion farmland by using a MAS-100 (Merck Inc.,USA) containing 20 mL of sterilized PAS at a height of 80 cm (Fig. 8b). Comparisons between biosampler IOM and biosampler MAS-100 on quantifications of *Acanthamoeba* by real-time PCR revealed that the Ct (cycle threshold) of sampling by IOM are more close to detection limit than MAS-100, results in the difficulty to determine *Acanthamoeba* concentrations. Therefore, MAS-100 was used as in the present study. In a real-time PCR assay a positive reaction is detected by accumulation of a fluorescent signal. The Ct is defined as the number of cycles required for the fluorescent signal to cross the threshold (i.e. exceeds background level). Ct levels are inversely proportional to the amount of target nucleic acid in the sample (i.e. the lower the Ct level the greater the amount of target nucleic acid in the sample). The sampling flow rate was 100 L/m and sampling time was 180 min, resulting in sampling volume of 18 m^3. Sterilizes PAS was refilled to 20 mL every 15 min during sampling. After sampling, the

PAS was transferred into a sterile centrifuge tube (50 mL) and transported under refrigeration to the laboratory within two days.

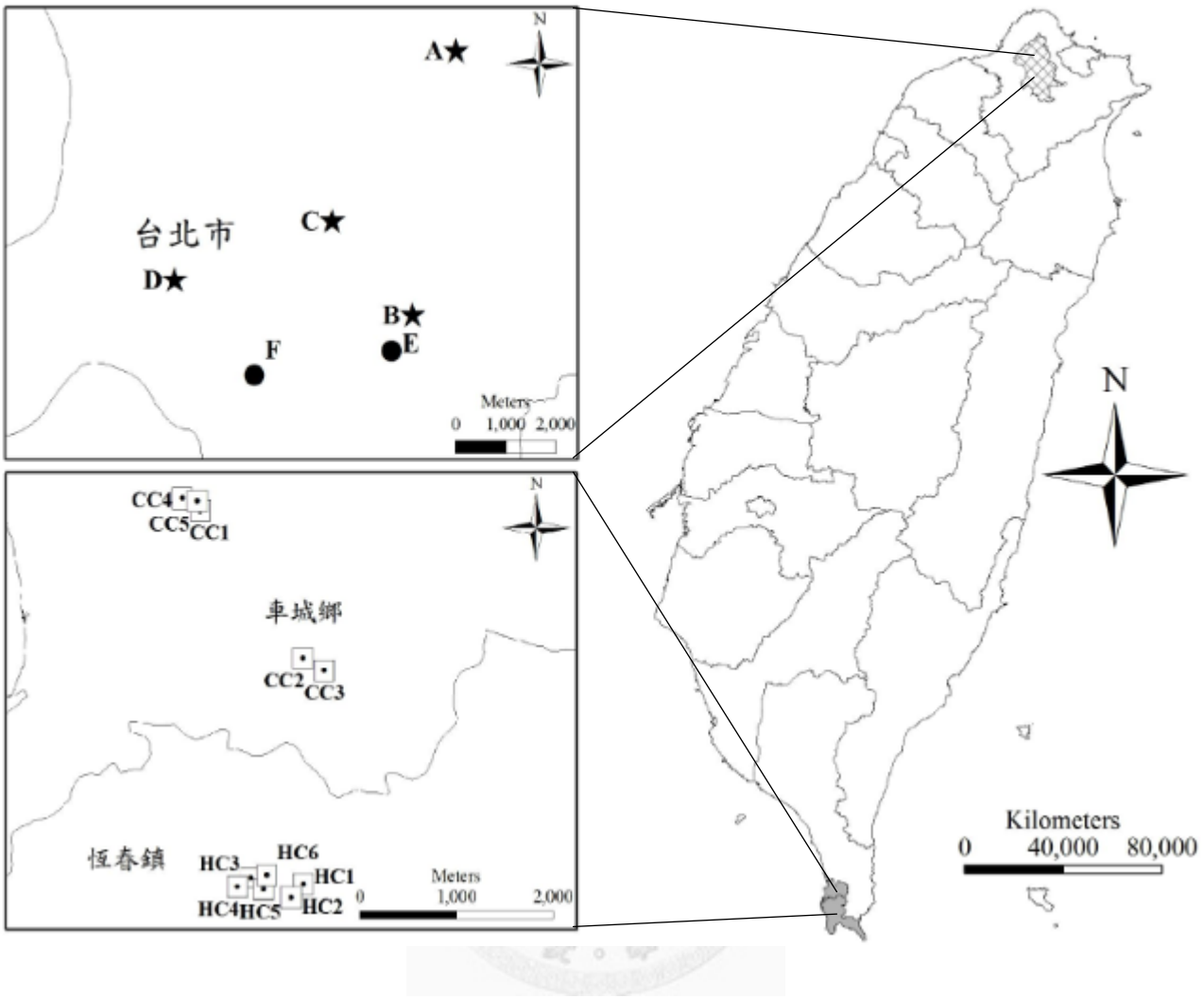


Figure 7 Sampling sites in present study. Star represents sampled community-type swimming pools and circle represents sampled school-type swimming pools in Taipei City. Square represents sampled onion farmlands in Pingtung County, Taiwan (HC: Hengchun Township and CC: Checheng Township).

(a) (b)

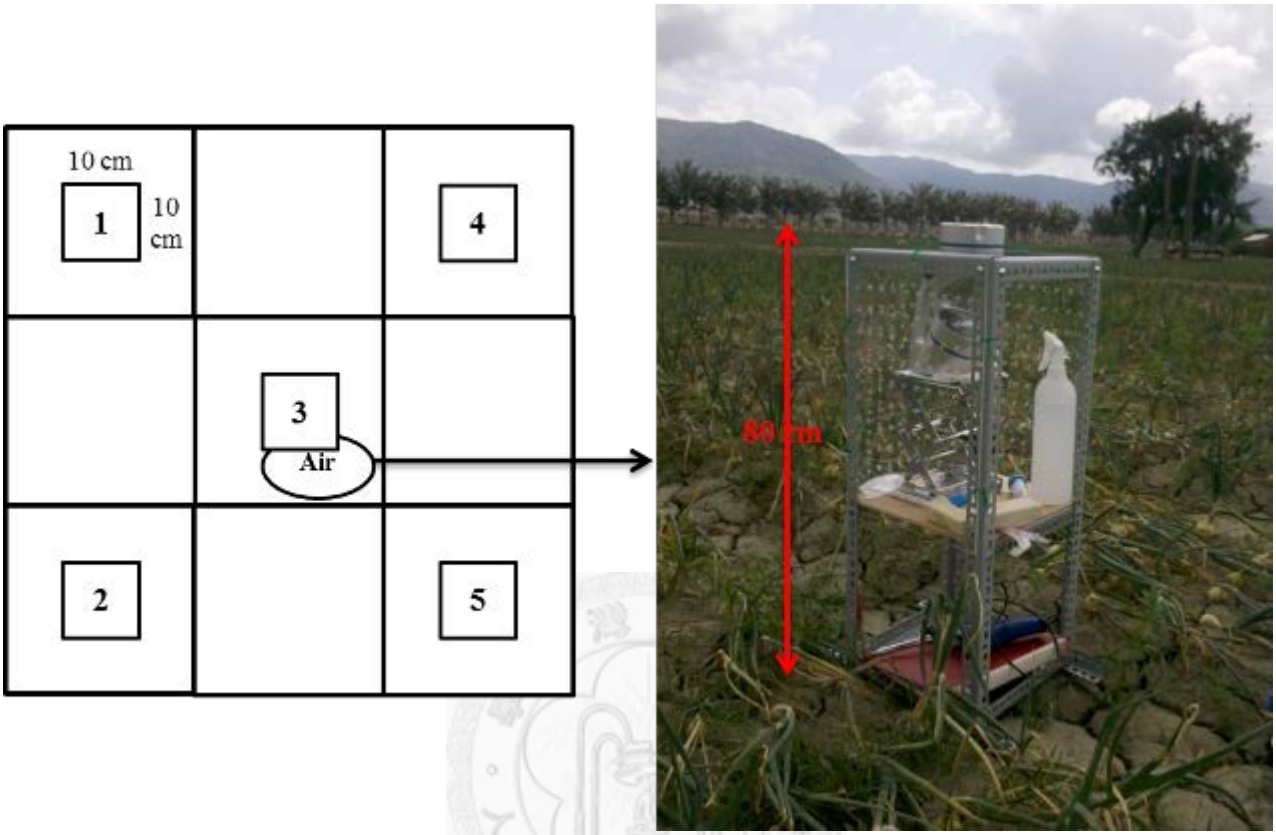


Figure 8 Illustration of soil (a) and air (b) sampling. (a) Square with number are the five area of soil samples collected. (b) Air sampling was conducted at the center of each onion farmland by using a MAS-100 (Merck Inc., USA) containing 20 mL of sterilized PAS at a height of 80 cm.

4.5. Pretreatment of environmental samples

4.5.1. Water samples from swimming pools

Each water sample (three L each) was filtered at a flow rate of 100 mL/30 sec through 1.2 μm

pore size RTTP Isopore Filter (47mm, Millipore, MA, USA). The filtration was stop when three mL of water sample remained above the membrane and filter concentrate (300 μL) was transfer into sterile 50 mL centrifugal tube with filter membrane and vortex for five seconds. The filter concentrate was distributed evenly over the surface of an NNA/heat-killed *E. coli* plate in duplicate. Besides, filter membrane was divided into eight pieces and each of pieces was placed face down onto the surface of an NNA/heat-killed *E. coli* plate. The plates were placed base upwards, sealed with Parafilm and incubated at 30°C. The filter membrane was removed from the NNA/heat-killed *E. coli* plate 3 days after the inoculum, sealed with Parafilm and incubated at 30°C.

4.5.2. Soil samples from onion farmland

Soil samples (ca. 300 – 500 g) were sieved by 10 standard mesh (two mm) in order to removing non-soil particles. Soil samples (ca. two g) were suspended in 20 mL of sterile distilled water (Kilic et al., 2004) and agitated at 75 rpm for one hour. An aliquot (100 μL) of each sample was inoculated onto NNA plates seeded with heat-killed *E.coli*. The plates were placed base upwards, sealed with Parafilm and incubated at 30°C.

4.5.3. Air samples from onion farmland

One mL of air sample was well-mixed by hand flapping the Eppendorf and inoculated on NNA

plates with heat-killed *E.coli.* The plates were placed base upwards, sealed with Parafilm, incubated at 30^{o}C.

4.6. Isolation and purification of amoeba

Daily inspection of environmental samples on NNA plates of water, soil and air samples was carried out by inverted phase microscopy (Eclipse TE2000-U, Nikon, Tokyo, Japan) at a magnification of 200X until any amoeboid plaque resembling amoeba, amoeba size and morphological features were according to Pussard and Pons (1977). The genus *Acanthamoeba* was considered based on two obvious characters, i.e. cyst size (five to 20 μm) and two layers of cyst forms (ectocyst and endocyst). Amoebae resembling clones were sub-cultured by cutting roughly 0.5 cm in diameter of agar containing amoebae, transferring the plaque centrally onto a fresh NNA/heat-killed *E. coli* plate. The clones of resembling amoeba were then maintained by passaging to another NNA/heat-killed *E.coli.* This procedure was repeated until amoebae cultures were free of contamination, which was determined by observing the presence of clones resembling amoeba on the NNA exclusively. The plates were considered as amoeba-negative if there was no amoeboid plaque in 14-day post-inoculation at 30^{o}C.

4.7. Xenic culture of amoeba

Xenic cultures were obtained by harvesting purified-isolate of amoeba from the plate cultures by picking up amoeba off the agar plates with a sterile scalpel and transferring the cells into T-25 tissue flask (Corning Incorporated Life Sciences, NY, USA) containing five mL sterile PAS medium with 1 mL heat-killed *E.coli* that had been already suspended in sterile PAS. The T 25 tissue flasks were incubated at 30^{o}C for seven days. The isolate of amoeba were examined once a week, and add with one mL heat-killed *E.coli*. The above process was applied for water, soil and air samples.

4.8. DNA extraction

The 2.5 mL of xenic culture (section 4.7) containing approximately 10^6 organisms, determined by a hemocytometer, were concentrated by centrifugation at 14000 x g for 10 minutes (Nagyova et al., 2010). The DNA of concentrated samples was extracted by using FastDNA®spin kit for soil (MP biomedical, Solon, OH, USA) following the manufacturers' instructions. The detailed procedures were described as following: The pellets were added with 978 µL of sodium phosphate buffer in Lysing matrix E tubes and mixed with 122 µL of MT buffer solution contained in FastDNA®spin kit for soil (MP biomedical, Solon, OH, USA). The tubes were placed in the FastPrep® Instrument (MP biomedical, Irvine, CA, USA) to homogenize and to disrupt the cells by bead-beating at a speed setting of 5.5 for one minute. After the tubes were centrifuged at 14000 x g

for 30 seconds at 25°C to pellet debris, the supernatants were transferred to the clean 1.5 mL centrifugal tubes. The Protein Precipitation Solution (250 µL) contained in FastDNA®spin kit for soil, was then added into the tubes and well-mixed by shaking by hand for 10 times. The tubes were centrifuged again at 14000 x g for five minutes at 25°C and the supernatants were transferred to 15 mL centrifugal tubes. The Binding Matrix Suspension contained in FastDNA®spin kit for soil was well-mixed by hands before use and one mL of which was added into the 15 mL centrifugal tube that described above. The tubes were inverted by hand for two minutes to allow binding of DNA and then the tubes were left in a rack at room temperature for three minutes to allow settling of silica matrix. After the 500 µL of supernatants were removed and discarded carefully by the pipette, the Binding Matrix in the remaining amount of supernatant were re-suspended by hands, and 600 µL of the mixture were transferred to the SPIN™ Filters which were contained in FastDNA®spin kit for soil. After centrifugation at 14000 x g for one minute at 25°C, the catch tubes were emptied soon afterwards. The remaining mixtures were added to the SPIN™ Filters and were centrifuged at 14000 x g for one minute at 25°C. The catch tubes were emptied again. Afterwards, 500 µL of prepared SEWA-M with adding 100 mL of 100% ethanol, which was contained in FastDNA®spin kit for soil, was added to the SPIN™ Filter, followed by centrifugation at 14000 x g for one minute at 25°C. After the catch tubes were emptied, the SPIN™ Filters were centrifuged at 14000 x g for 2 minutes at 25°C without any addition of liquid to dry the matrix of residual wash solution. The

catch tubes were discarded and replaced by the new 1.5 mL centrifugal tubes. After the centrifugal tubes were air-dried in the Biological Safety Cabinet II at room temperature for five minutes, 100 μL of DNase/Pyrogen-Free Water (DES, contained in FastDNA®spin kit for soil) were then well-mixed with the Binding Matrix above the SPIN™ Filter by pipetting. Finally, the tubes were centrifuged at 14000 x g for one minute at 25°C to elute 100 μL of DNA into the clean 1.5 mL centrifugal tubes. The DNA was ready to use and stored at –20°C.

In addition to environmental samples, 2.5 mL of *Acanthamoeba polyphaga* (ATCC 50492) incubated in ATCC medium 712 at 25°C for three days were also processed for DNA extraction and used as positive control in the following polymerase chain reaction (PCR) (Bio Rad iCycler, Philadelphia, USA) amplification and gel electrophoresis.

4.9. Polymerase chain reaction (PCR) amplification

Primers of JDP1 (5'-GGCCCAGATCGTTTACCGTGAA-3') and JDP2 (5'-TCTCACAAGCTGCTAGGGAGTCA-3') were used to amplify the ASA.S1 region of the gene (*Rns*) coding for the amoeba's nuclear small-subunit ribosomal RNA (Schroeder et al., 2001). A 423- to 551-bp *Acanthamoeba*-specific amplimer ASA.S1 fragment allows specific detection of *Acanthamoeba* since it is discriminating for the genus and can be obtained from all known 18S rDNA genotypes (Schroeder et al., 2001). PCR was performed in a volume of 25 μL containing five

μL of DNA, 12.5 μL of 2X Taq PCR Master Mix (Genomics) and 2 μL of 10 μM of each primer (Mission Biotech). The amplification reaction was carried out in a PCR under the following thermal conditions: 95^{o}C for 10 min, followed by 35 cycles of 94^{o}C for one min, 58^{o}C for one min and 72^{o}C for one min, with a final elongation step of 10 min at 72^{o}C (Liang et al., 2010).

4.10. Gel electrophoresis

When the 1.5 % agarose gel (SeaKem® LE Agarose, Rockland, USA) was ready, the dams and the comb were removed carefully. The 400 mL of 1X TAE buffer was poured in the reaction box. The 25 μL of the PCR amplification product samples (including environmental samples and positive control (*Acanthamoeba polyphaga* (ATCC 50492)) and negative controls (sterilized ddH_2O) were mixed with four μL of loading dye (Sigma-Aldrich (G 7654), Saint Louis, USA). The 10 μL of 100-bp DNA ladder (Sigma-Aldrich, Saint Louis, Missouri, USA) was mixed with 2 μL of loading dye. The samples, DNA ladder, positive control and negative control were placed into adjacent wells by using an Eppendorf pipette. Samples were electrophoresing at 50 V for 90 minutes. The gel was stained with 0.5 μM/ml ethidium bromide (Sigma-Aldrich (SI-E1385), USA) in two L for 10 min, and the gel was observed under a UV-light transilluminator (Universal hood, Bio-Rad, Philadelphia, USA). The unit was operating by the quantity one acquisition window on your computer, select Live/Focus mode and adjusts your image position, size, focus, and intensity

using the lens controls. After the image is optimized, the image was captured. A typical procedure is described below. The Universal Hood II was switched on and starts the quantity one software on computer. The gel was positioned in the Universal Hood II and pressed the Epi-Illumination button to turn on the Epi White lights. The gel was centered on the transilluminator platen and closes the door. Adjust the lens Iris, Zoom, and Focus while looking at the computer screen. The gel was re-position if necessary. The image was acquiring by pressing the appropriate light source for your sample and when a satisfactory image is seen click Freeze.

4.11. DNA sequencing

A single band at 423- to 551-bp PCR products represents as *Acanthamoeba* isolates were cut off by scalpel. Following procedures on DNA purification and sequencing using the JDP1 primer were conducted by the company of Genomic (Taipei, Taiwan). Prior to DNA sequencing, all PCR products were cleaned with the Purification kit (Genomics). The DNA gel extraction of samples was extracted following the manufacturers' instructions. The gel with DNA of samples was put with EB (elution buffer) at 65^{o}C water bathing. The 300 μL of binding buffer for every 100 mg of agarose gel were added and the gel mixture were incubated at 60^{o}C for five min until the gel slice is completely dissolved. The solution was poured to a fresh adsorption column and centrifuged at 13000 rpm for one min and the liquid in the collection tube was poured off. The 600 μL washing

buffer (WB) was added before centrifugation at 13000 rpm for one min and the liquid was pour off the into beaker, and centrifuged at 13000rpm for 10 min. The column was put into a fresh EP tube and 30-50 μL elution buffer (EB) was added in order to elute the DNA. The five μL of the eluted sample was used to identify with electrophoresis. *Acanthamoeba*-specific primer JDP1 allows specific detection of *Acanthamoeba*. Sequencing was performed with one μL BigDye terminator V3.1, 100 ng to500 ng plasmid or 20 to 50ng PCR product (depends on size), 3.2 pmol primer, 3.5 μL 5x buffer and nucelase-free H_2O up to 20 μL. The amplification reaction was carried out in a An automated DNA sequencers ABI 3730XL sequencer (Applied Biosystems, Foster City, CA) under the following thermal conditions: 25 cycles of 90^{o}C for 10 sec, 50^{o}C for five sec and 60^{o}C for four min, with a final elongation step at 4^{o}C.

4.12. Identification of *Acanthamoeba* species

In order to identify the species of 42*Acanthamoeba* isolates obtained from swimming pools and onion farmlands, 42 sequences of 18S rDNA gene of isolates were aligned by the Basic Local Alignment Search Tool (BLAST) program of the US National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST) to search for the most similar sequences. The nucleotide blast was chosen, and the DNA sequence derived from environment isolates (section 4.11.) were entered the in enter query sequence. Others (nr etc.) was chose on choose search set and

somewhat similar sequences (blastn) was choose for program selection, and BLAST in the bottom of the page was entered, then the results were shown. The highest percentage similarity was taken to identify the species. Species identifications are compared the 42 sequences of 18S rDNA gene of isolated strains were aligned by the BLAST program to search for the most similar sequences. The highest percentage similarity was taken to identify the species, with highest max score, highest total score, highest query coverage, lowest E Value (typically, $E < 0.05$), highest max ident. Following are the description of above-mentioned index in nucleotide sequence in BLAST and used to identify the DNA sequences. Max score is the calculation from the number of matches and gaps and the higher relative to the query length is better. Total score is the sum of the score of all (high scoring pairs, HSPs) from the same database sequence. Query coverage is the percent of the query sequence matched by the database entry and is the length coverage of the input query sequence by different HSPs from the same database sequence. E Value is the number of entries required in the database for a match to happen by random chance, and smaller E Values are better and typically, $E < .05$ is required to be considered significant. Max ident is the percent that the genes match up within the limits of the full match (e.g. deletions or additions base-pair reduce this value).

4.13. Morphological properties of isolated *Acanthamoeba*

Once the sequence of *Acanthamoeba* isolates from environments identified as *Acanthamoeba*

in BLAST, the *Acanthamoeba* isolates were pictured under an inverted microscope at 1000X.

4.14. Phylogenetic analysis and genotyping

In order to categorize the *Acanthamoeba* isolates of the present study, 18S rDNA sequences of 12 *Acanthamoeba* (Table 9) were retrieved from GenBank. Since the preliminary alignments revealed our isolates in the present study to be most similar to T4, T5 and T15 genotypes, more representatives of these three groups were included in the comparison, most of the remaining genotypes being not included in the final comparison.

All sequence alignments of isolated *Acanthamoeba* were performed by constructing a neighbour-joining distance tree using the Mega 4.0.2 program (Molecular Evolutionary Genetic Analysis software, ver. 4.0.2) with 1,000 bootstrapped replicates. Isolated strains that had identical DNA sequences by BLAST analysis were clustered into the same group in order to strengthen the bootstrap value to avoid the phylogenetic tree in failure of distinguishing same sequence from each other. Altogether, there were 19 groups in present study. These 19 *Acanthamoeba* sequence groups were aligned with 12 published *Acanthamoeba* sequence types obtained from BLAST (as reference strains, Table 9). Genotypic classifications of all *Acanthamoeba* isolates were performed by assembling and comparing the ASA.S1 sequences of these isolates with reference sequences encompassing currently established genotypes of *Acanthamoeba*. Genotypic classifications of all

Acanthamoeba isolates were done in MEGA4 with neighbor-joining tree by assembling and comparing the ASA.S1 sequences of 19 isolate groups in present study with reference sequences encompassing currently established genotypes of AK and GAE. The same cluster represents the same genotype in neighbor-joining tree.

Table 9 18S rDNA sequences of *Acanthamoeba* as reference strains

Reference sequence					
No.	Genotype	Species classification	Strain	Abbreviation	GenBank assession number
1	T4	*A. polyphaga*	Nagington, 1501/3D	T4 - A. polyphaga (AF019062)	AF019062
2	T4	*A. mauritaniensis*	1652 ATCC:50253	T4 - A. mauritaniensis (AY351647)	AY351647
3	T4	*A. sp.*	ATCC 50497	T4 - A. sp. (U07410)	U07410
4	T4	*A. castellanii*	Neff ATCC 50373	T4 - A. castellanii (U07416)	U07416
5	T4	*A. royreba*	Oak Ridge ATCC 30884	T4 - A. royreba (U07417)	U07417
6	T5	*A. lenticulata*	strain 45 ATCC:50703	T5 - A. lenticulata (U94730)	U94730
7	T5	*A. lenticulata*	72/2	T5 - A. lenticulata (U94732)	U94732
8	T5	*A. lenticulata*	118; 25-1, ATCC50706	T5 - A. lenticulata (U94736)	U94736
9	T5	*A. lenticulata*	strain JC-1 ATCC:50428	T5 - A. lenticulata (U94739)	U94739
10	T5	*A. lenticulata*	PD2S; ATCC 30841	T5 - A. lenticulata (U94741)	U94741
11	T5	*A. lenticulata*	407-3A NJSP-3-2	T5 - A. lenticulata (U94734)	U94734
12	T15	*A. jacobsi*	AC194	T15 - A. jacobsi (AY262362)	AY262362

4.15. Thermotolerance and osmotolerance testing on Environmental *Acanthamoeba* isolates

4.15.1. Criteria for selecting samples for thermotolerance and osmotolerance testing

Samples from different sampling sites and categorized into different genotypes were chosen for thermotolerance and osmotolerance testing. In addition, two isolates were taken for testing if there were more than two isolates obtained from the same sampling site and in the same genotype. On the other hand, the sample was taken if there is only 1 isolate in same sampling sites and in the same genotype.

4.15.2 Preparation of testing samples for thermotolerance and osmotolerance

In order to form trophozoites, one mL of heat-killed *E.coli* suspension was added to the xenic cultures of environmental samples, incubated in T25 tissue flasks at 30^{o}C for three days. Cell suspensions were transferred to the 15 mL sterile centrifugation tubes by aseptic autopipetting, and centrifuged at 200 x g for eight minutes at 4^{o}C to collect trophozoites (Grimm *et al.*, 2001). Cell pellets were re-suspended in one mL of sterilize PAS and the amoebic concentration were determined by a hemocytometer (Marienfeld, Lauda-Konigshofen, Germany). With appropriate volume, the suspension was serially diluted with sterile PAS to make the cell suspensions at a concentration of 10^3 trophozoites in 10 μL of PAS. The samples were ready for thermotolerance and osmotolerance testing.

4.15.3. Thermotolerance

An aliquot of 10 µL of tested samples (10^3 trophozoites) was transferred to the center of a fresh 1.5% NNA plate pre-covered with heat-killed *E. coli* suspension, sealed with parafilm and waited for 2 hours in the laminar flow to allow settling the *Acanthamoeba* on agar surface. The plates were then incubated at 30°C (as control), 37°C, and 42°C for 10 days and at 52°C for 24 hours prior to incubation at 30°C for nine days. The experiments were conducted in triplicate. In addition, *A. polyphaga* (ATCC 30461) and *A.castellanii* (ATCC30234) were used as potentially pathogenic strain and probably pathogenic, respectively. With *A. polyphaga* (ATCC 30461) and *A.castellanii* (ATCC30234) were used as potentially pathogenic strain and probably pathogenic strain, respectively. *Acanthamoeba polyphaga* (ATCC30461), obtained from the American Type Culture Collection, was originally isolated from human corneal scrapings of a case of AK (Visvesvara et al., 1975) and therefore identified as potentially pathogenic strain in the present study. *A. castellanii* (ATCC30234) was obtained from the American Type Culture Collection, originally from yeast culture, United Kingdom (Visvesvara and Balamuth, 1975). The cytotoxicity determined using LDH assays revealed 51.5 ± 4.5 reported by Maghsood et al. in 2005, and thus recognized *A. castellanii* (ATCC30234) as probably pathogenic strain.

4.15.4 Osmotolerance

The 10 µL of tested samples were transferred to the center of a fresh 1.5% NNA plate (at 10^3 trophozoites / heat-killed *E.coli*/NNA agar plate) containing no mannitol (as control), 0.5 M mannitol or 1 M mannitol. The NNA plates were pre-covered with heat-killed *E. coli* suspension. After inoculation of *Acanthamoeba*, the plates were sealed with parafilm and waited for two hours in the laminar flow to settle *Acanthamoeba* on the agar surface. The plates were incubated at 30^{o}C for 10 days, the experiments were done in triplicate. *A. castellanii* (ATCC30234) was obtained from the American Type Culture Collection, originally from yeast culture, United Kingdom (Visvesvara and Balamuth, 1975). The cytotoxicity determined using LDH assays revealed 51.5 ± 4.5 reported by Maghsood et al. in 2005, and thus recognized *A. castellanii* (ATCC30234) as probably pathogenic strain.

4.15.5. Evaluation of the level of *Acanthamoeba* growth subject to thermotolerance and osmotolerance testing

All the assays were done in triplicated. After inoculation for 10 days, the number of *Acanthamoeba* trophozoites and cysts on NNA plates were counted under an inverted microscope at 100X for nine microscope fields located at the distance of 15 to 25 mm from the center of each plate. The mean numbers of *Acanthamoeba* in nine microscope fields of triplicate plats were

determined. The mean number/microscopic field was converted into semi-quantitative scores, i.e. score as number of *Acanthamoeba*/microscopic field counts of zero being scored as ─, one to14 being scored as +, 15 to 29 being scored as 2+, 30 to 59 being scored as 3+, 60 to 89 being scored as 4+, 90 to 149 being scored as 5+, 150 to 299 being scored as 6+, 300 to 500 being scored as 7+, respectively (n=3) (Caumo et al., 2009).

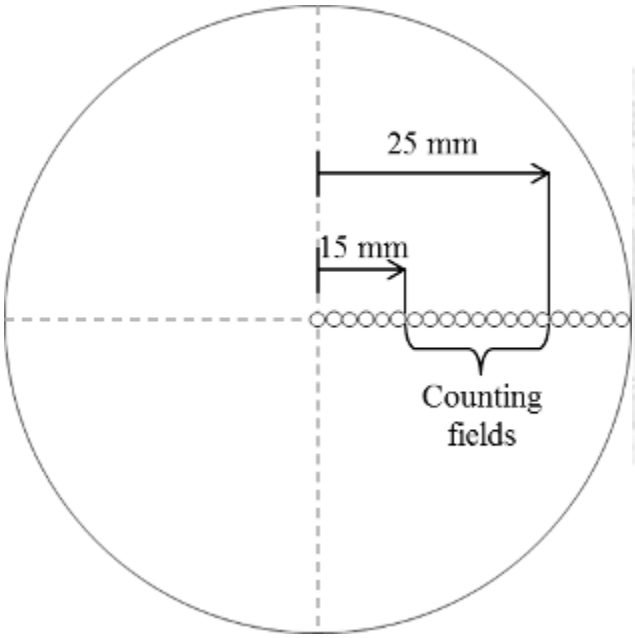


Figure 9 Illustration of evaluations on thermotolerance and osmotolerance

4.16. Measurement of environmental factors

4.16.1. Environment factors for water samples of swimming pools

Free residual chlorine, pH, water temperature, turbidity, hardness, dissolved oxygen, salinity,

conductivity, total dissolved solids in pool water were measured in situ by the direct-reading instruments on sites. The above environmental factors were measured in six replicates. In addition, water samples were analyzed back to the laboratory for determination of the heterotrophic plate count, which were measured in triplicate.

4.16.1.1. Free chlorine

The free chlorine was measured by the Pocket colorimeter (Hach Company, Loveland, Co., Ohio, USA). The DPD Free Chlorine Reagent was well-mixed with 10 ml of water samples, which were then placed in the dark for one minute. After reading of the Pocket colorimeter was set to zero using the deionized water, the pool water sample reacted with DPD reagent was measured to obtain the level of free chlorine.

4.16.1.2. pH

The pH value of water sample was measured by HACH sensION 156 portable multi-parameter instrument (SensION 156, Hach Co., Loveland, Co., Ohio, USA). Before each use, the reading was calibrated by putting the cleaned electrode into the standard solutions of pH 10.0, pH 7.0, and pH 4.0 and pushing the enter bottom until reading values were stable. A calibration curve between pH 4 and pH 10 was built automatically by the pH meter, according to the operation manual of Hach

sensION156. Once the pH meter was calibrated, the pH values of test samples were determined by placing the electrode into the samples and waiting for stable reading values.

4.16.1.3. Water temperature

The water temperature was measured by HACH sensION 156 portable multi-parameter instrument (SensION 156, Hach Co., Loveland, Co., Ohio, USA). The probe of the Hach sensION156 was into water samples and the temperature was shown.

4.16.1.4. Turbidity

The turbidity was measured by the Hach 2100P Turbidimeter (Hach Company, Loveland, Co., Ohio, USA). The instrument was calibrated every three months following the procedures described below: After the instrument was set on calibration mode, four Formazin primary standards with different values of turbidity (< 0.1, 20, 100 and 800 NTU) were used to calibrate the 2100P Turbidimeter. After the environmental water samples were added into the cuvette and mixed without bubbles, the cuvettes were measured by the instrument directly.

4.16.1.5. Hardness

The hardness of water samples was measured by the Hardness meter (HANNA instruments,

Inc., Woonsocket, RI, USA). The procedures were described below:

The probable or expected concentration range of the test samples was chosen (Low Range: 0-250 ppm; Medium Range: 200-500 ppm; High Range: 400 to 750 ppm). Afterwards, the water sample (0.5 mL) was put into a glass cuvette containing 10 ml of medium-concentration indicator reagent contained in Hardness Reagent A-B (HANNA), followed by adding two drops of Hardness Buffer Reagent B contained in the Hardness Reagent A-B. The solution was mixed well and the Hardness meter was set to zero. After a package of Hardness Reagent C (Ion Specific Meters Reagents Set, HANNA) was added, the hardness was measured in two minutes.

4.16.1.6. Dissolved oxygen

The dissolved oxygen of water samples was measured by HACH sensION 156 portable multi-parameter instrument (SensION 156, Hach Co., Loveland, Co., Ohio, USA). The sension156 was calibrated to read the dissolved oxygen in water as 100% saturation. The instrument was then ready to use. The probe of the Hach sensION156 for dissolved oxygen measurement was into water samples and the dissolved oxygen was shown.

4.16.1.7. Salinity

The conductivity of water samples was measured by HACH sensION 156 portable

multi-parameter instrument (SensION 156, Hach Co., Loveland, Co., Ohio, USA). This instrument was calibrated by selecting the calibration range and putting the electrode into the calibration solution with known conductivity value (i.e., 1000 μS/cm). The instrument was then calibrated automatically and ready to use. The calibration was automatically by transferred the value of conductivity unit into salinity unit.

4.16.1.8. Conductivity

The conductivity of water samples was measured by HACH sensION 156 portable multi-parameter instrument (SensION 156, Hach Co., Loveland, Co., Ohio, USA). This instrument was calibrated by selecting the calibration range and putting the electrode into the calibration solution with a known conductivity value (i.e., 1000 μS/cm). The instrument was then calibrated automatically and ready to use.

4.16.1.9. Total dissolved solids (TDS)

The conductivity of water samples was measured by HACH sensION 156 portable multi-parameter instrument (SensION 156, Hach Co., Loveland, Co., Ohio, USA). This instrument was calibrated by selecting the calibration range and putting the electrode into the calibration solution with known conductivity value (i.e., 1000 μS/cm). The instrument was then calibrated

automatically and ready to use. The calibration was automatically by transferred the value of conductivity unit into TDS unit.

4.16.1.10. Heterotrophic Plate Count

Water samples (1L) containing 1 ml of $Na_2S_2O_3$ (10 %) collected from swimming pool was thoroughly mixed by rapidly making about 25 complete up-and-down (or back-and-forth) movements. Samples were diluted with sterile deionized water 10-fold. After thorough mix of all dilutions, an aliquot (200 µL) of undiluted and diluted samples were spreaded onto the surface of R2A in duplicates by using a sterile bent glass rod. Once the inoculum was absorbed completely, the plates were incubated upside down at 28^{o}C for seven days. Colony-forming units (CFUs) were then counted (Reasoner and Geldreich, 1985).

The concentrations of heterotrophic plate count from swimming pool were determined by the standard method for the examination of wastewater and water of American Public Health Association (APHA, 2005) (21st Edition, Section 9215. Heterotrophic Plate Count. American Public Health Association, Washington, D.C., 2005).

4.16.2. Environment factors for soil samples of onion farmlands

Water content, pH and heterotrophic plate count of soil samples were determined by Mr.

Chung-Long Kuo in the laboratory. Data were collected during sampling at 2012.

4.16.2.1. Water content

Soil samples were weighted to 3 g onto tin foil paper (W1), the container was place with soils in oven and dry at 105°C for 48 hours to obtain a constant weight (W2), the moisture content of the soils were calculated as a percentage of the dry soil weight in triplicate (Rodriguez-Zaragoza et al., 2005) using the following equations:

Water content (%) = (W1 ─ W2)/W2 * 100

4.16.2.2. pH

Soils in 20 g of were suspended in 20 mL water were mixed and placed for one hour at room temperature. The pH values of water contained soils were measured by HACH sensION 156 portable multi-parameter instrument with probe was put into soil suspended in water (SensION 156, Hach Co., Loveland, Co., USA). The pH values of test samples were determined by placing the electrode into the samples and waiting for stable reading values.

4.16.2.3. Heterotrophic Plate Count

Samples were transported under refrigeration to the laboratory (Huang and Hsu, 2010). Soil

samples were diluted with 10 mL sterile PAS by 10^6 to 10^8 folds. All dilutions were thoroughly mixed by rapidly making about 25 complete up-and-down (or back-and-forth) movements. An aliquot (100 µL) of original samples and each dilution were spreaded onto R2A in duplicates. Plates were incubated upside down at 28^{o}C for seven days. Colony-forming units (CFUs) were counted at the end of the incubation.

4.16.3. Environment factors for air samples of onion farmlands

Temperature, wind velocity, and relative humidity (RH%) of the atmosphere were measured each time at the beginning and 30 min, 60 min, 90 min, 120 min and 150 min by the VELOCICALC Air Velocity Meter (9545, Shoreview , USA). The average of six measurement data was calculated and illustrates the environmental factors in air. The VELOCICALC Air Velocity Meter was facing the wind and parallel with the MAS-100, and the data-log time was set one min. Thus, the value for a given sampling time was determined as of environmental factors in farmlands was performed by Mr. Chung-Long Kuo. Data were collected during sampling at 2012.

Chapter 5 Results

5.1. Quality assurance and quality control on thermotolerance and osmotolerance

5.1.1. Distance of *Acanthamoeba* ATCC strains migrated after two hours inoculum onto NNA/heat-killed *E.coli*

To evaluate the distance of *A. polyphaga* (ATCC 30461) and *A.castellanii* (ATCC 30234) migrated at initial inoculum at 0 M mannitol and 1 M mannitol NNA/heat-killed *E.coli*; the results were quantitatively through enumeration *Acanthamoeba* under inverted microscopic at 100X at each microscopic field. Table 10 indicated number of trophozoite or cyst for *A. polyphaga* and *A.castellanii* on each microscopic field. The farthest migration after two hours initial inoculum of *Acanthamoeba* ATCC strains was *A.castellanii* (ATCC 30234) on 0 M for two hours (n=1), revealed two trophozoite or cyst seen at the distance 9.45 mm in 8th microscopic field. Migration of *Acanthamoeba* in each testing were occurred once the *Acanthamoeba* trophozoite and cyst presence at area out of first inoculation, Therefore, the trophozoite or cyst of *Acanthamoeba* seen at the distance larger than 9.45 mm at the end of incubation periods was attributed to their migration (Fig. 10). Here, we assumed presence of *Acanthamoeba* on the distance larger than 14.18 mm with 12th microscopic field were attributed to *Acanthamoeba* growth and migration ability.

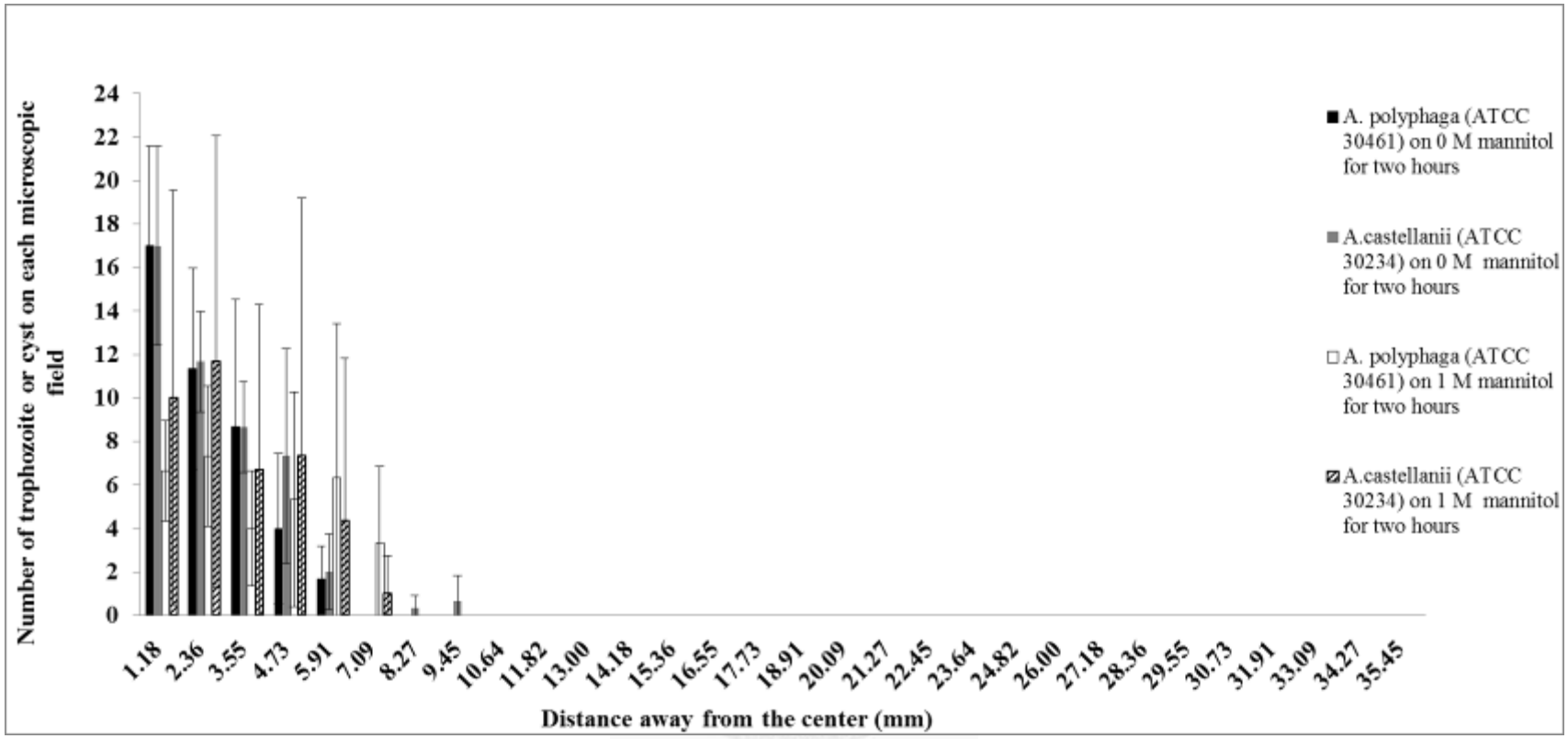


Figure 10 Distance of *Acanthamoeba* ATCC strains migrated after two hours inoculum onto NNA/heat-killed *E.coli*

5.1.2. Thermotolerance

Evaluation on the distance of *A. polyphaga* (ATCC 30461) and *A.castellanii* (ATCC 30234) migrated during proliferation at various incubation temperatures, were enumerated *Acanthamoeba* cells under an inverted microscope at 100X distance between 15.36 and 35.45 mm away from plate center. Table 10 and Table 11 present the mean number of *A. polyphaga* and *A.castellanii* cells grew and observed at 30 ^{o}C to 52^{o}C. At 30^{o}C, the highest mean number *A. polyphaga* cells (142 cells/microscopic field) were revealed at 6th day of incubation at the distance between 26.00 and 35.45 mm away from the plate center (Table 10 and Fig. 11). As for *A.castellanii*, at 30^{o}C, the highest mean number (69 cells/microscopic field) was observed at the distance between 15.36 and 24.82 mm away from the plate center (Table 11 and Fig. 12).

A. polyphaga and *A.castellanii* were also able to grow at 37^{o}C. The highest number of *A. polyphaga* (69 cells/microscopic field) growth was found at 9th day of incubation at the distance between 15.36 and 24.82 mm away from the plate center (Table 10 and Fig. 13). As for *A.castellanii*, the highest number was found at 8th day of incubation at the distance between 26.00 and 35.45 mm away from the plate center, which were 54 cells/microscopic field (Table 11 and Fig. 14).

Neither *A. polyphaga* nor *A.castellanii* grow and migrate at 42^{o}C (Table 10 and Table 11). To investigate the effects of higher temperatures on *Acanthamoeba* viability, cysts of *A. polyphaga* and *A.castellanii* were exposed 52 ^{o}C for 24 hours, followed by incubation at 30^{o}C to favor excystation.

However, no migration or growth was found for both test cells (Table 10 and Table 11).

Table 10 Thermotolerance pretesting of *Acanthamoeba polyphaga*

Distance away from the center (mm)[a]	Incubation temperature	Mean number of *A.polyphaga* cyst and trophozoite / microscopic field[b]									
		Day after incubation									
		1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th
15.36 ~ 24.82	30°C	2.44 ± 3.57	12.04 ± 13.8	64.85 ± 62.1	65.93 ± 32.39	72.26 ± 39.3	129.11 ± 102.98	76.26 ± 46.36	78 ± 52.19	77.15 ± 52.13	76.04 ± 48.29
15.36 ~ 24.82	37°C	0.19 ± 0.40	1.96 ± 6.95	4.37 ± 12.99	27.22 ± 52.45	47.26 ± 50.63	42.44 ± 29.6	42.85 ± 17.66	39.67 ± 19.12	69.07 ± 54.47	63.85 ± 68.18
15.36 ~ 24.82	42°C	0	0	0	0	0	0	0	0	0	0
15.36 ~ 24.82	52°C (24 hours), followed by 30°C[c]	0	0	0	0	0	0	0	0	0	0
26.00 ~ 35.45	30°C	0.41 ± 0.89	1.81 ± 2.68	13.56 ± 16.86	61.63 ± 47.12	64.85 ± 18.59	141.59 ± 89.55	69.78 ± 29.46	66.74 ± 17.13	68.19 ± 27.69	69.07 ± 28.07
26.00 ~ 35.45	37°C	0.15 ± 0.53	0	0	0	4.33 ± 15.75	16.59 ± 25.01	32.26 ± 21.43	43.78 ± 22.28	38.52 ± 14.39	30.19 ± 9.27

26.00 ~ 35.45	42°C	0	0	0	0	0	0	0	0	0	0
26.00 ~ 35.45	52°C (24 hours), followed by 30°C[c]	0	0	0	0	0	0	0	0	0	0

[a] Distance away from the center of incubated NNA agar plate with the unit in mm.

[b] Mean number of *A. polyphaga* cyst and trophozoite (n=3).

[c] Incubation at 52°C for 24 hours, followed by incubation at 30°C for 9 days.

Table 11 Thermotolerance pretesting of *Acanthamoeba castellanii*

Distance away from the center (mm)[a]	Incubation temperature	Mean number of *A.castellanii* cyst and trophozoite / microscopic field[b]									
		Day after incubation									
		1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th
15.36 ~ 24.82	30°C	1.59 ± 3.19	13.96 ± 13.12	69.15 ± 57.34	46.56 ± 14.52	42.93 ± 18.59	56.48 ± 26.76	33.37 ± 10.63	31.15 ± 11.14	48.63 ± 20.93	45.04 ± 15.01
15.36 ~ 24.82	37°C	0.00	1.22 ± 2.41	10.93 ± 12.92	22.44 ± 13.63	33.81 ± 12.92	35.52 ± 10.16	26.33 ± 9.17	36.44 ± 13.26	29.30 ± 10.51	29.19 ± 8.15

15.36 ~ 24.82	42°C	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
15.36 ~ 24.82	52°C (24 hours), followed by 30°C[c]	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
26.00 ~ 35.45	30°C	0.00	0.00	5.37 ± 8.01	41.11 ± 47.09	62.11 ± 36.46	61.89 ± 28.6	57.22 ± 33.84	51.44 ± 21.37	61.22 ± 30.70	55.93 ± 29.93
26.00 ~ 35.45	37°C	0.00	0.00	0.22 ± 0.7	0.85 ± 1.75	7.93 ± 7.85	25.07 ± 15.41	26.44 ± 17.31	53.59 ± 22.23	24.59 ± 16.74	27.67 ± 16.67
26.00 ~ 35.45	42°C	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
26.00 ~ 35.45	52°C (24 hours), followed by 30°C[c]	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

[a] Distance away from the center of incubated NNA agar plate with the unit in mm.

[b] Mean number of *A. castellanii* cyst and trophozoite (n=3).

[c] Incubation at 52°C for 24 hours, followed by incubation at 30°C for 9 days.

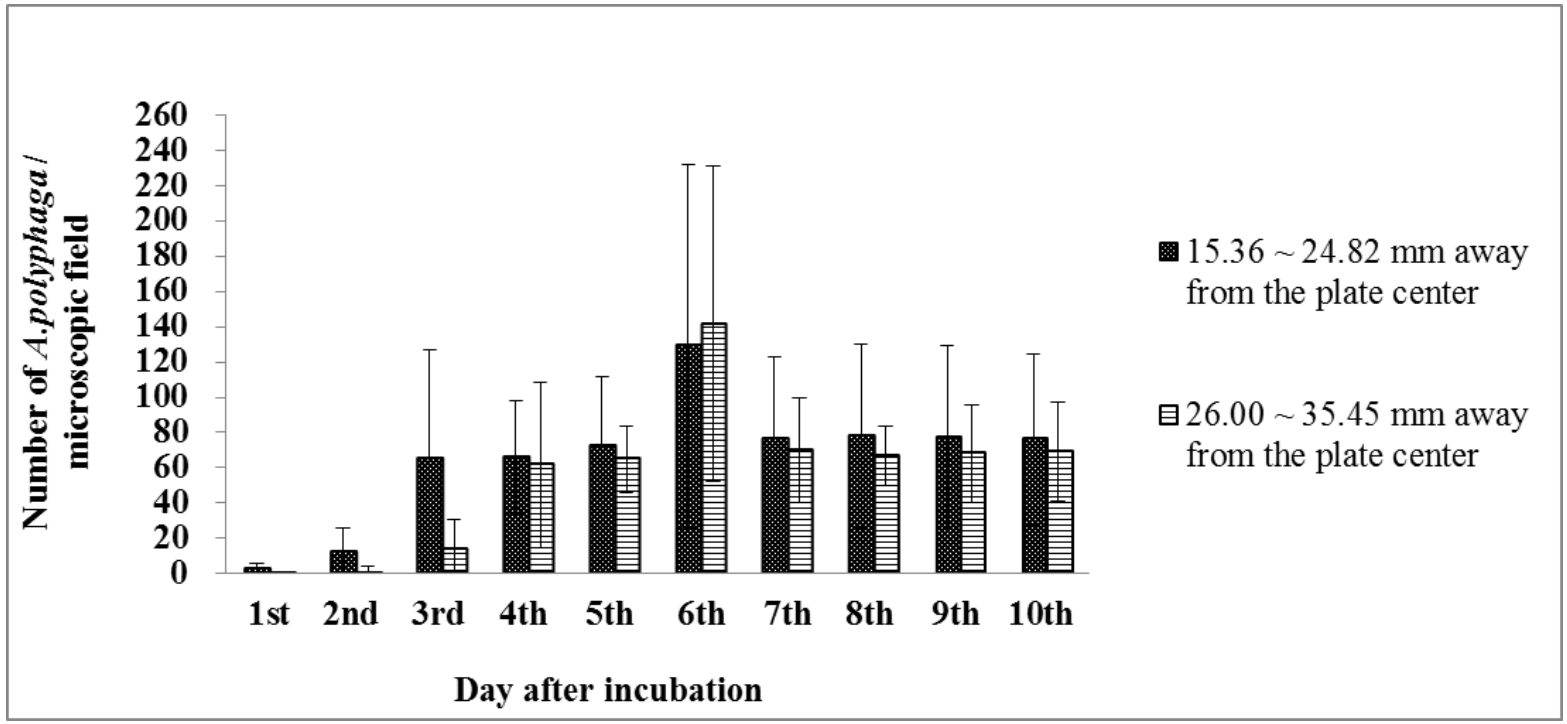


Figure 11 Number of *A.polyphaga* at 30°C and 0M mannitol incubation on each day (n=3).

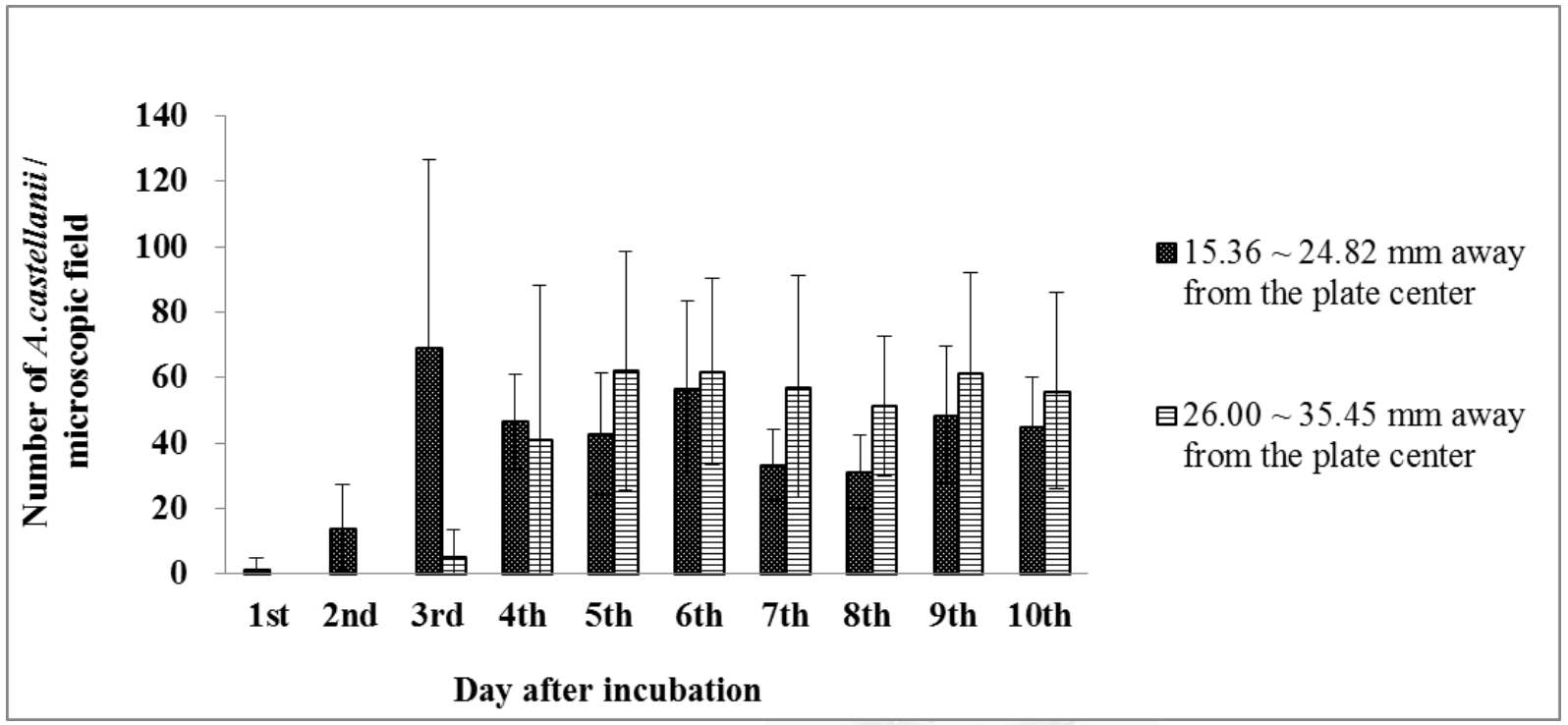


Figure 12 Number of *A.castellanii* at 30°C and 0M mannitol incubation on each day (n=3)

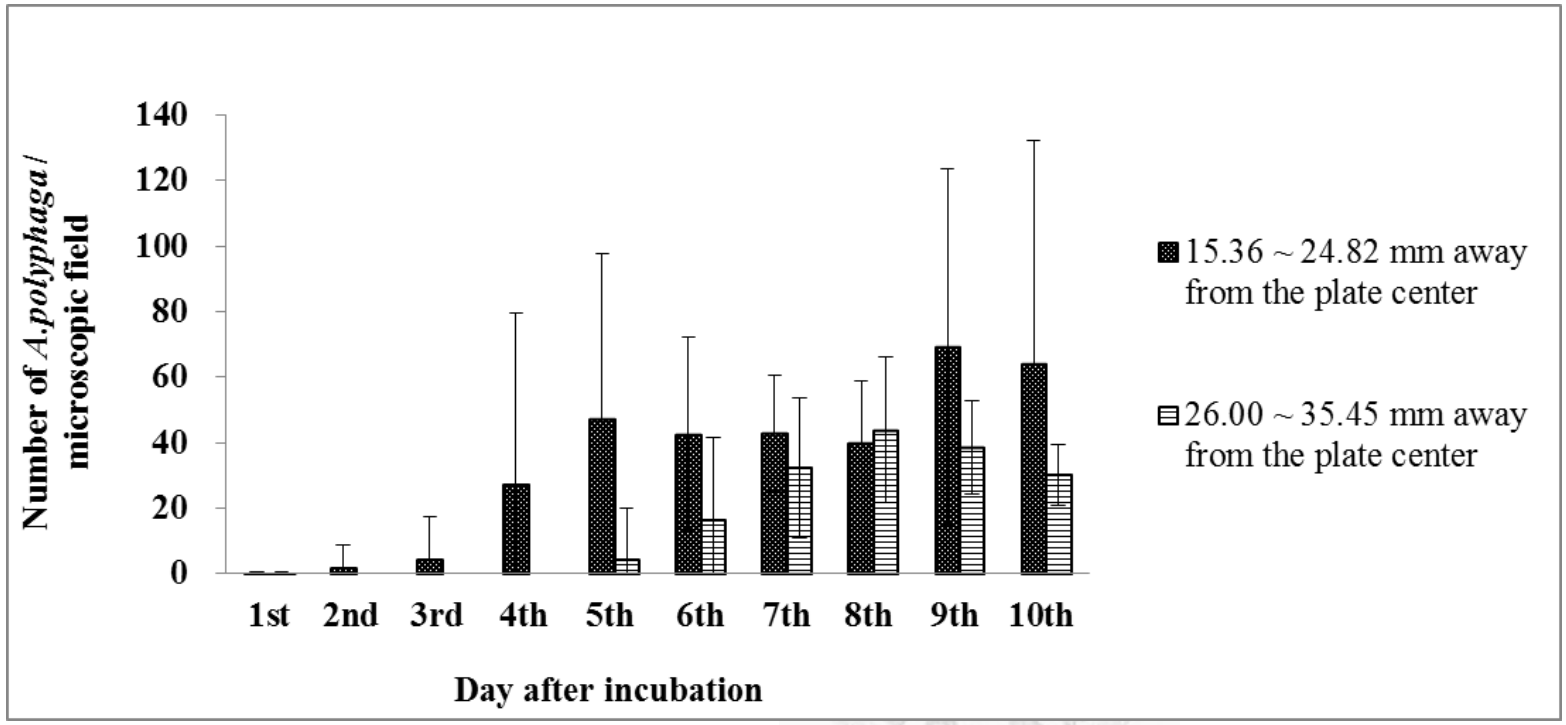


Figure 13 Number of *A.polyphaga* at 37°C and 0M mannitol incubation on each day (n=3).

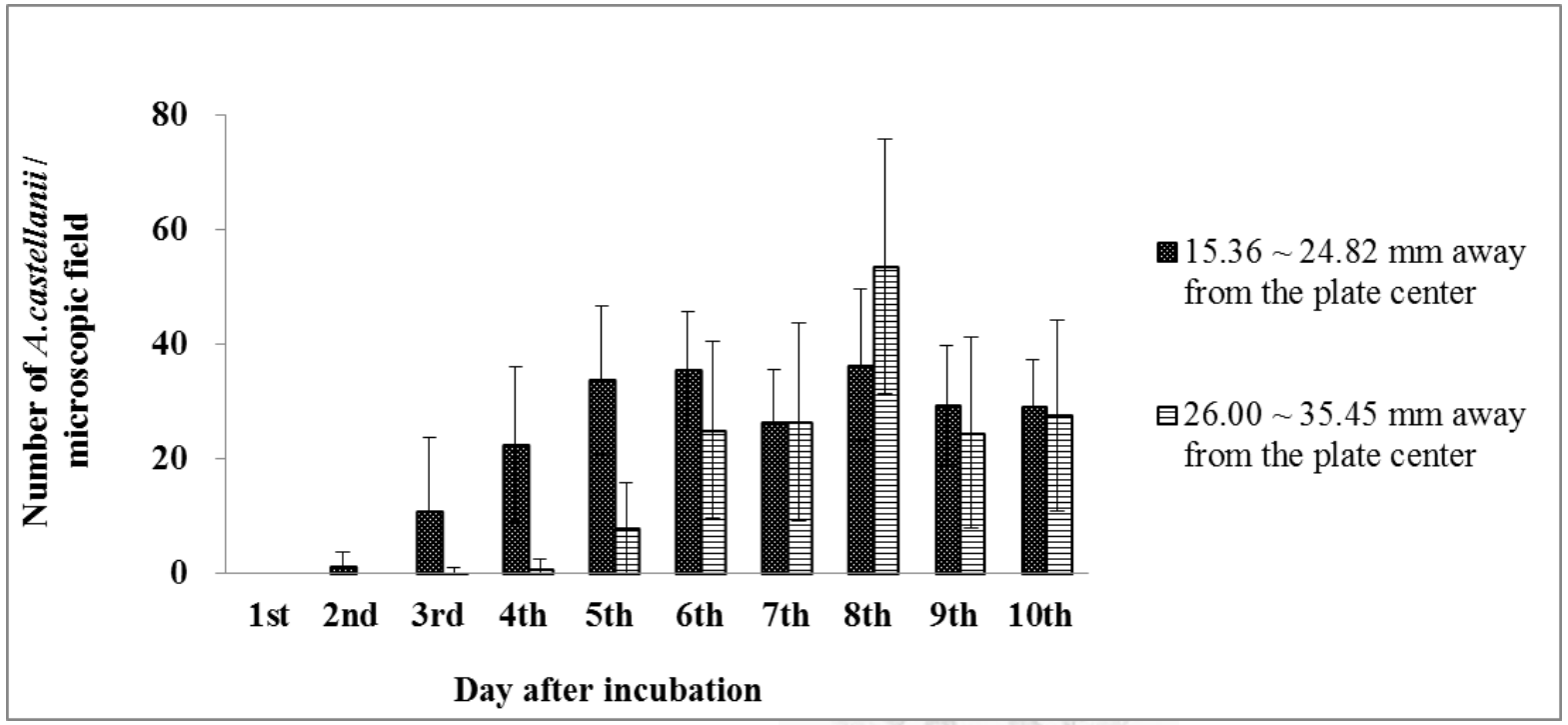


Figure 14 Number of *A.castellanii* at 37°C and 0M mannitol incubation on each day (n=3)

5.1.3. Osmotolerance

To evaluate the distance of *A. polyphaga* (ATCC 30461) and *A. castellanii* (ATCC 30234) migrating during proliferation at 0 M and 1 M mannitol at 30^{o}C, the number of *Acanthamoeba* was enumerated under an inverted microscope at 100X. Table 12 and Table 13 demonstrated the ability of *A. polyphaga* and *A. castellanii* to proliferate on plates without mannitol. The maximum number of *A. polyphaga* (142 cells/microscopic field) occurred at 6th day of incubation at the distance of 26.00 to 35.45 mm away from the center plate (Table 12) As for *A.castellanii*, the maximum number (69 cells/microscopic field) occurred at 3th day of incubation at the distance of from 15.36 and 24.82 mm away from the center plate (Table 13). *A. polyphaga* grew better than *A. castellanii* at 1M mannitol as the maximum number of *A. polyphaga* (78 cells/microscopic field) was found at 10th day of incubation at the distance of 15.36 and 24.82 mm away from the center plate (Table 12 and Fig. 15). *A. castellanii* with the maximum number of six cells/microscopic field on 10th day of incubation at the distance of 15.36 and 24.82 mm (Table 13 and Fig. 16). Besides, lower numbers of *A. castellanii* in plates containing 1 M mannitol was consist with observed during the 10th day compared to *A. polyphaga* (Table 12 and Table 13).

Table 12 Osmotolerance pretesting of *Acanthamoeba polyphaga*

Distance away from the center (mm)[a]	Osmolarity of NNA	Mean number of *A.polyphaga* cyst and trophozoite / microscopic field[b]									
		Day after incubation at 30°C									
		1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th
15.36 ~ 24.82	0 M mannitol	2.44 ± 3.57	12.04 ± 13.8	64.85 ± 62.1	65.93 ± 32.39	72.26 ± 39.3	129.11 ± 102.98	76.26 ± 46.36	78 ± 52.19	77.15 ± 52.13	76.04 ± 48.29
15.36 ~ 24.82	1 M mannitol	0.67 ± 2.06	0.30 ± 0.91	3.15 ± 7.49	10.78 ± 22.22	24.41 ± 36.49	34.70 ± 38.45	44 ± 26.69	77 ± 46.55	72.93 ± 33.8	78.07 ± 41.36
26.00 ~ 35.45	0 M mannitol	0.41 ± 0.89	1.81 ± 2.68	13.56 ± 16.86	61.63 ± 47.12	64.85 ± 18.59	141.59 ± 89.55	69.78 ± 29.46	66.74 ± 17.13	68.19 ± 27.69	69.07 ± 28.07
26.00 ~ 35.45	1 M mannitol	0.00	0.00	0.00	0.11 ± 0.58	0.52 ± 1.42	6.07 ± 18.01	6.41 ± 12.84	24.59 ± 40.81	25.63 ± 42.81	36.30 ± 45.35

[a] Distance away from the center of incubated NNA agar plate with the unit in mm.

[b] Mean number of *A. polyphaga* cyst and trophozoite (n=3).

Table 13 Osmotolerance pretesting of *Acanthamoeba castellanii*

Distance away from the center	Osmolarity of NNA	Mean number of *A.castellanii* cyst and trophozoite / microscopic field[b]
		Day after incubation at 30°C

(mm)[a]		1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th
15.36 ~ 24.82	0 M mannitol	1.59 ± 3.19	13.96 ± 13.12	69.15 ± 57.34	46.56 ± 14.52	42.93 ± 18.59	56.48 ± 26.76	33.37 ± 10.63	31.15 ± 11.14	48.63 ± 20.93	45.04 ± 15.01
15.36 ~ 24.82	1 M mannitol	0.00	0.00	0.00	0.00	0.70 ± 1.71	1.26 ± 2.21	1.96 ± 2.33	2.41 ± 2.86	3.48 ± 3.41	5.78 ± 6.36
26.00 ~ 35.45	0 M mannitol	0.00	0.00	5.37 ± 8.01	41.11 ± 47.09	62.11 ± 36.46	61.89 ± 28.6	57.22 ± 33.84	51.44 ± 21.37	61.22 ± 30.70	55.93 ± 29.93
26.00 ~ 35.45	1 M mannitol	0.00	0.00	0.00	0.00	0.00	0.00	0.04 ± 0.19	0.00	0.89 ± 1.95	1.41 ± 2

[a] Distance away from the center of incubated NNA agar plate with the unit in mm.

[b] Mean number of *A. castellanii* cyst and trophozoite (n=3).

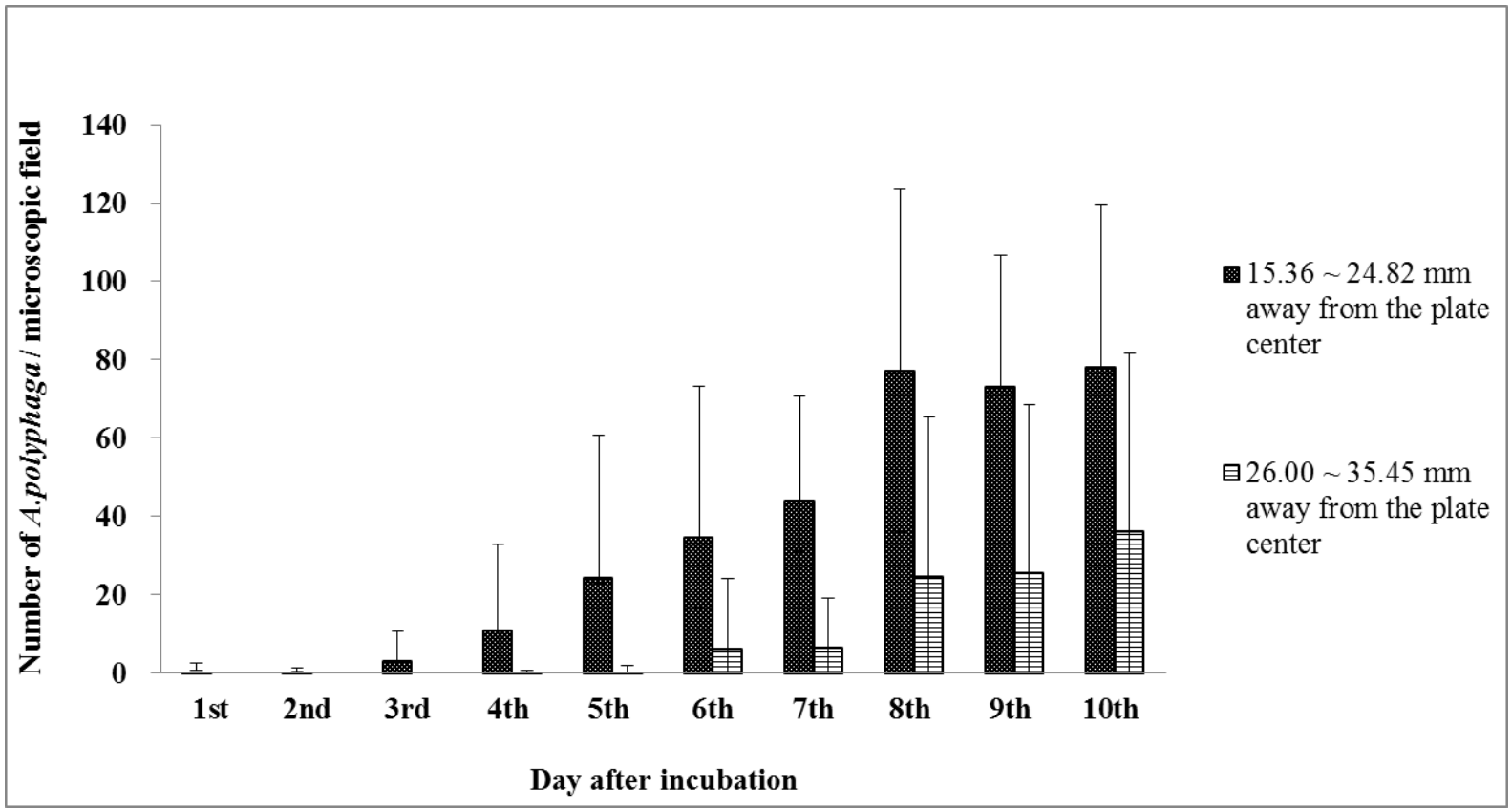


Figure 15 Number of *A.polyphaga* at 1 M mannitol incubation on each day (n=3)

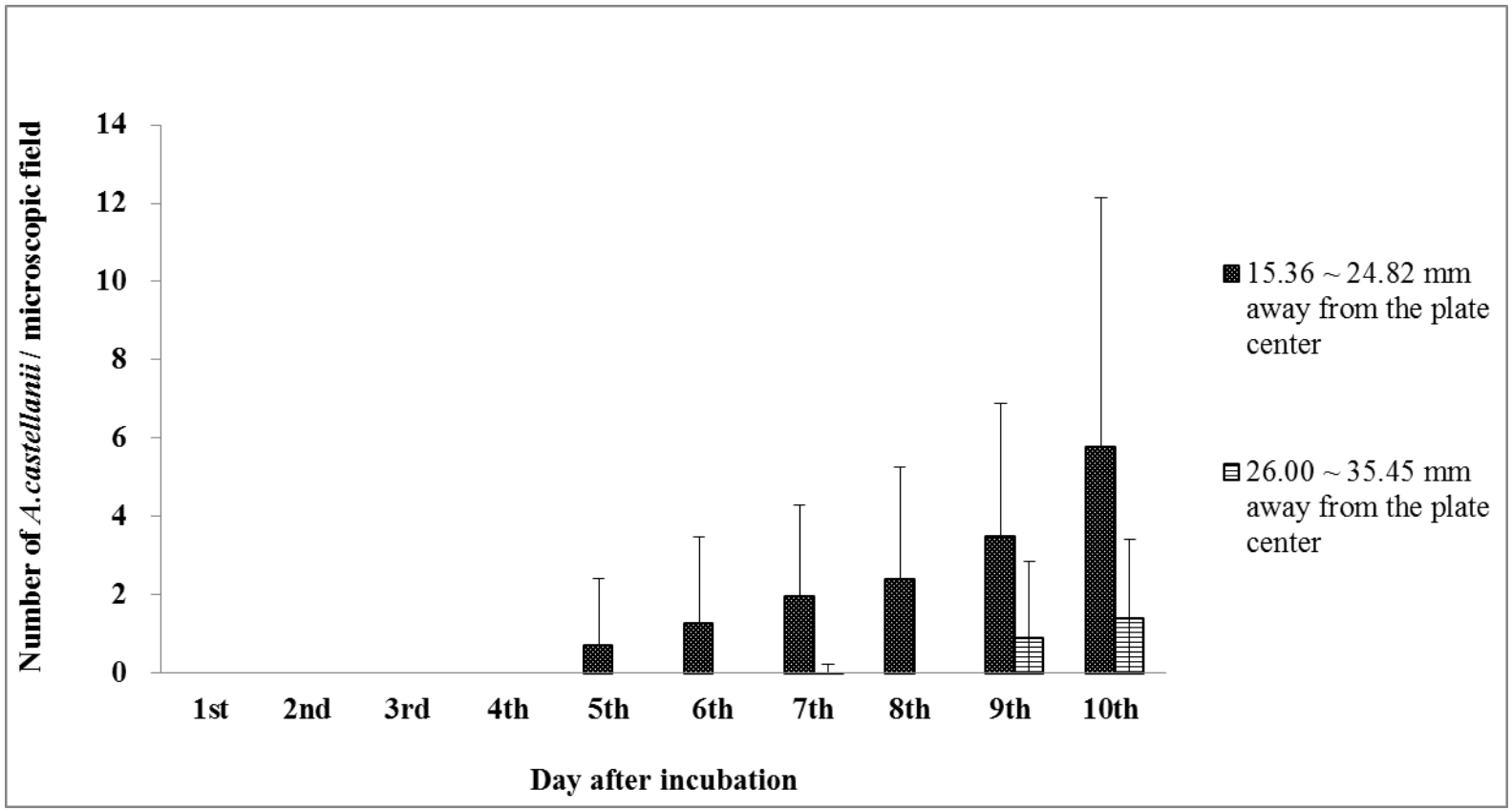


Figure 16 Number of *A.castellanii* at 1 M mannitol incubation on each day (n=3)

5.1.4. Evaluations the level of growth on thermotolerance and osmotolerance

The growth level of *Acanthamoeba* ATCC strains on 0 M mannitol at 30 ^{o}C were used as control groups in thermotolerance and osmotolerance pretesting since the incubation condition were the most favorable for *Acanthamoeba* growth. The level of growth were based on thermotolerance incubated at 37^{o}C, 42 ^{o}C, 52 ^{o}C for 24 hours and at 30 ^{o}C for 9 days and osmotolerance incubated on 1 M mannitol at 30 ^{o}C. Under thermotolerance incubated at 37^{o}C, 42 ^{o}C, 52 ^{o}C for 24 hours and at 30 ^{o}C for 9 days and osmotolerance incubated on 1 M mannitol at 30 ^{o}C condition, the migration of *Acanthamoeba* ATCC strains were slower than *Acanthamoeba* ATCC strains growth on 0 M mannitol at 30^{o}C. Therefore, the level of growth were evaluated by thermotolerance incubated at 37^{o}C, 42^{o}C, 52^{o}C for 24 hours and at 30^{o}C for 9 days and osmotolerance incubated on 1 M mannitol at 30^{o}C.

At 37^{o}C, the maximum growth was 69.07 mean numbers of *A.polyphaga* cyst and trophozoite per microscopic field at 9th and 10th day incubation and at a distance between 15.36 and 24.82 mm away from the center of NNA plates; The maximum growth was 63.85 mean numbers of *A.castellanii* cyst and trophozoite per microscopic field at 8th day and at a distance between 26.00 and 35.45 mm away from the center of NNA plates. There were no *A.polyphaga* and *A.castellanii* cells incubated at 42^{o}C and 52^{o}C. When it comes to *Acanthamoeba* ATCC strains incubated on 1M mannitol at 30^{o}C, the maximum growth was 78.07 mean numbers of *A.polyphaga* cyst and

trophozoite per microscopic field at 10th day incubation and at a distance between 15.36 and 24.82 mm away from the center of NNA plates; The maximum growth was 5.78 mean numbers of *A.castellanii* cyst and trophozoite per microscopic field at 10th day incubation and at a distance between 15.36 and 24.82 mm away from the center of NNA plates. The maximum growth were 10th day incubation and at a distance between 15.36 and 24.82 mm away from the center of NNA plates besides *A.castellanii* growth at 37°C. Considerations on the consistence in days after incubation and at the distance away from the center of NNA plates, the 10th day incubation and at a distance between 15.36 and 24.82 mm away from the center of NNA plates were used in *Acanthamoeba* isolates from environments.

5.2. *Acanthamoeba* isolated from sampling sites

5.2.1. Positive rate of *Acanthamoeba*

Twelve water samples from six thermal swimming pools and 14 soil and 14 air samples from 11 onion farmlands were collected. However, a soil sample (HC2FARM Soil Sample2) from Hengchun farmland 2 was loss during the transportation. Thus, there were 13 soil samples analyzed in the present study.

Suspected amoeba were obtained from environmental sample inoculated onto NNA/heat-killed *E.coli*, for which the *Acanthamoeba* resembling clone was transferred into new NNA/heat-killed

E.coli through daily examination under inverted microscope (section 4.6. Isolation and purification of amoeba). Identification of *Acanthamoeba* was conducted through observation by an inverted microscopic, PCR with *Acanthamoeba* genus specific amplimers ASA.S1, and DNA sequencing with *Acanthamoeba* genus specific primer JDP1. There were total 42 *Acanthamoeba* isolates obtained in the present study (Table 14). In detail, there were five in A swimming pool, eight in E swimming pool, three in F swimming pool, five in B swimming pool, three in C swimming pool and two in D swimming pool. Besides, each soil sample obtained one *Acanthamoeba* isolate and three *Acanthamoeba* were isolated from 14 air samples (Table 14). In terms of positive rate, *Acanthamoeba* were detected in 12 out of 12 water samples collected (100%), 13 out of 13 soil samples (100%), and three out of 14 air samples (21.43%), which were from Hengchun (farmlands 4, 3 and 6) (Table 14).

Table 14 Positive rate of *Acanthamoeba* isolates from swimming pools and onion farmlands

Sample ID	City	Location	Sampling site	Sample type	Sampling date (Day/Month/Year)	*Acanthamoeba* isolate ID[a]	Positive rate (%)[b]
ASP Water Sample1	Taipei City, Taiwan	Swimming pool	Swimming pool A	water	7/2/2011	A_ i4	100 (2/2)
						A_ i5	
ASP Water Sample2						A_ i6	
						A_ i7	
						A_ i8	
ESP Water Sample1	Taipei City, Taiwan	Swimming pool	Swimming pool E	water	7/16/2011	E_i1	100 (2/2)
						E_i2	
						E_i3	
ESP Water Sample2						E_i4	
						E_i5	
						E_i6	
						E_i7	
						E_i8	
FSP Water Sample1	Taipei City, Taiwan	Swimming pool	Swimming pool F	water	03/21/2012	F_i1	100 (2/2)

						F_i2	
FSP Water Sample2						F_i3	
BSP Water Sample1	Taipei City, Taiwan	Swimming pool	Swimming pool B	water	03/21/2012	B_i1	100 (2/2)
						B_i2	
BSP Water Sample2						B_i3	
						B_i4	
						B_i5	
CSP Water Sample1	Taipei City, Taiwan	Swimming pool	Swimming pool C	water	03/28/2012	C_i1	100 (2/2)
						C_i2	
CSP Water Sample2						C_i3	
DSP Water Sample1	Taipei City, Taiwan	Swimming pool	Swimming pool D	water	03/28/2012	D_i1	100 (2/2)
DSP Water Sample2						D_i2	
HC1FARM Soil Sample2	Pingtung County, Taiwan	Onion farmland	Hengchun Township, farmland 2	Soil	11/20/2011	HC1_Soil_s2	100 (1/1)

HC1FARM Soil Sample5	Farmland 5	Soil	11/22/2011	HC1_Soil_s5	100 (1/1)
HC2FARM Soil Sample1	Farmland 1	Soil	03/20/2012	HC2_Soil_s1	100 (1/1)
HC2FARM Soil Sample2	Farmland 2	Soil	03/20/2012	Sample loss	Sample loss[c]
HC2FARM Soil Sample3	Farmland 3	Soil	03/21/2012	HC2_Soil_s3	100 (1/1)
HC2FARM Soil Sample4	Farmland 4	Soil	03/21/2012	HC2_Soil_s4	100 (1/1)
HC2FARM Soil Sample5	Farmland 5	Soil	03/22/2012	HC2_Soil_s5	100 (1/1)
HC2FARM Soil Sample6	Farmland 6	Soil	03/22/2012	HC2_Soil_s6	100 (1/1)
CC1FARM Soil Sample1	Checheng Township, farmland 1	Soil	11/23/2011	CC1_Soil_s1	100 (1/1)

CC1FARM Soil Sample5	Farmland 5	Soil	11/23/2011	CC1_Soil_s5	100 (1/1)
CC2FARM Soil Sample1	Farmland 1	Soil	03/19/2012	CC2_Soil_s1	100 (1/1)
CC2FARM Soil Sample2	Farmland 2	Soil	03/23/2012	CC2_Soil_s2	100 (1/1)
CC2FARM Soil Sample3	Farmland 3	Soil	03/23/2012	CC2_Soil_s3	100 (1/1)
CC2FARM Soil Sample4	Farmland 4	Soil	03/24/2012	CC2_Soil_s4	100 (1/1)
HC1FARM Air Sample2	Hengchun Township, farmland 2	Air	11/20/2011	ND[d]	ND
HC1FARM Air Sample4	Farmland 4	Air	11/21/2011	HC1_Air_s4	100 (1/1)
HC2FARM Air Sample1	Farmland 1	Air	03/20/2012	ND	ND
HC2FARM Air	Farmland 2	Air	03/20/2012	ND	ND

Sample2					
HC2FARM Air Sample3	Farmland 3	Air	03/21/2012	HC2_Air_s3	100 (1/1)
HC2FARM Air Sample4	Farmland 4	Air	03/21/2012	ND	ND
HC2FARM Air Sample5	Farmland 5	Air	03/22/2012	ND	ND
HC2FARM Air Sample6	Farmland 6	Air	03/22/2012	HC2_Air_s6	100 (1/1)
CC1FARM Air Sample1	Checheng Township, farmland 1	Air	11/23/2011	ND	ND
CC1FARM Air Sample5	Farmland 5	Air	11/23/2011	ND	ND
CC2FARM Air Sample1	Farmland 1	Air	03/19/2012	ND	ND
CC2FARM Air Sample2	Farmland 2	Air	03/23/2012	ND	ND

CC2FARM Air Sample3	Farmland 3	Air	03/23/2012	ND	ND
CC2FARM Air Sample4	Farmland 4	Air	03/24/2012	ND	ND

[a] *Acanthamoeba* were isolated from the original sample inoculum onto NNA/heat-killed *E.coli* and transferred the *Acanthamoeba* resembling clone into new NNA/heat-killed *E.coli* through daily examination under inverted microscopic and followed by *Acanthamoeba* identification process

[b] Number of *Acanthamoeba* positive sample divided by number of the samples collected

[c] Sample loss during transportation

[d] ND non-detectable for *Acanthamoeba*

5.2.2. Morphological properties of isolated *Acanthamoeba*

The trophozoite and cyst of *Acanthamoeba* isolates are presented in figure 17 – 38.

(a) (b)

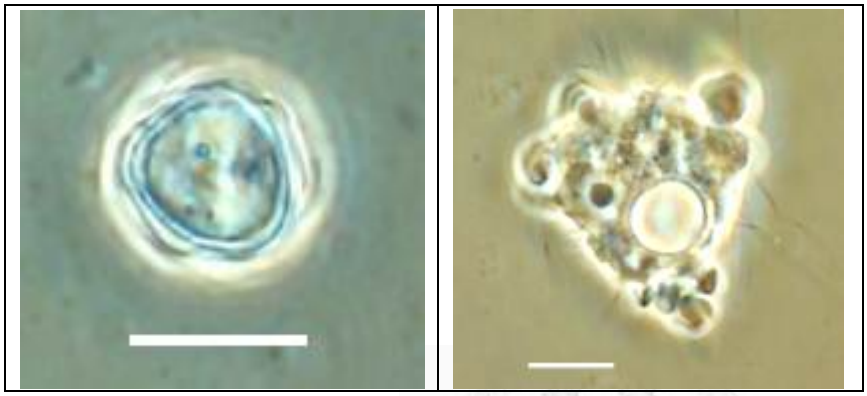


Figure 17 Trophozoite (a) and cyst (b) of *Acanthamoeba* isolate A_i4 viewed by an inverted microscope at a magnification of 1000X. (Bar = 10 µm).

(a) (b)

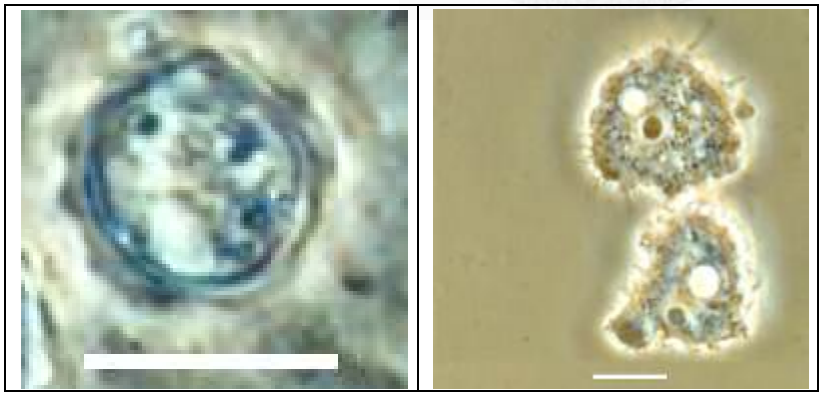


Figure 18 Trophozoite (a) and cyst (b) of *Acanthamoeba* isolate E_i1 viewed by an inverted microscope at a magnification of 1000X. (Bar = 10 µm)

(a) (b)

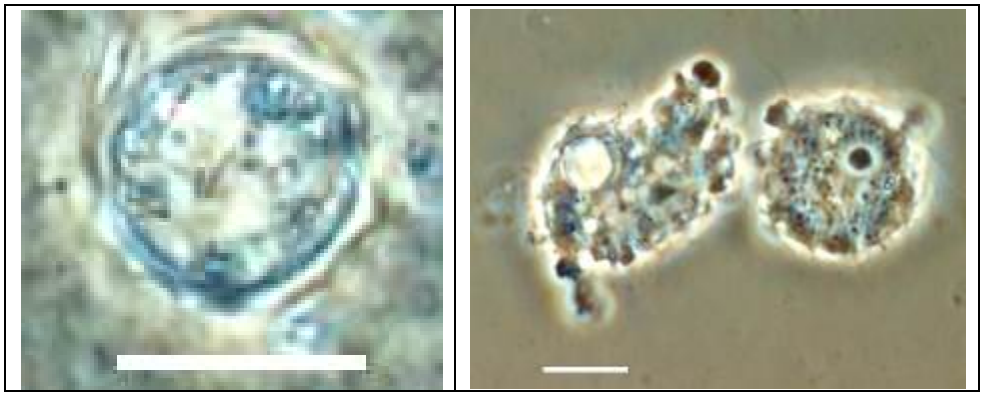


Figure 19 Trophozoite (a) and cyst (b) of *Acanthamoeba* isolate F_i1 viewed by an inverted microscope at a magnification of 1000X. (Bar = 10 µm)

(a) (b)

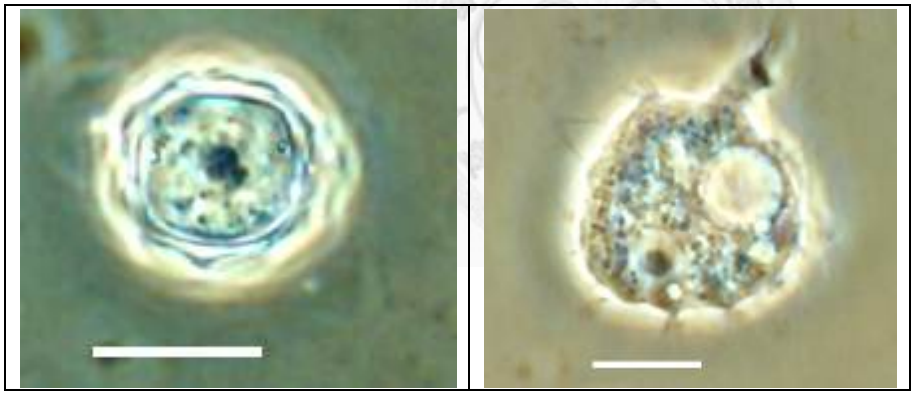


Figure 20 Trophozoite (a) and cyst (b) of *Acanthamoeba* isolate B_i1 viewed by an inverted microscope at a magnification of 1000X. (Bar = 10 µm)

(a) (b)

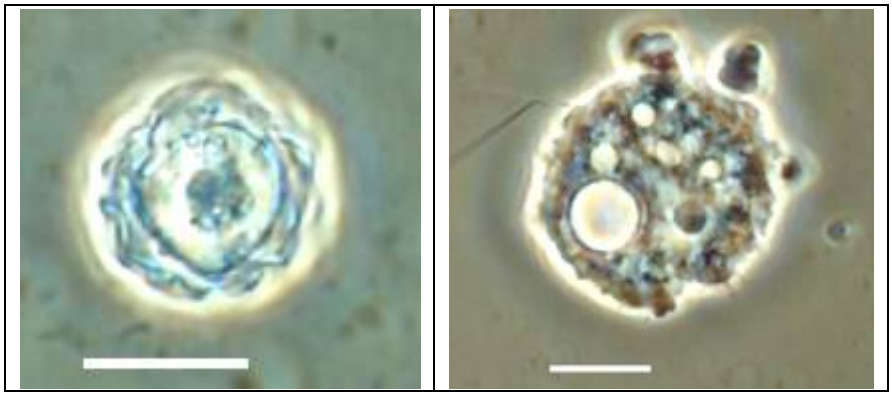


Figure 21Trophozoite (a) and cyst (b) of *Acanthamoeba* isolate C_i1 viewed by an inverted microscope at a magnification of 1000X. (Bar = 10 µm)

(a) (b)

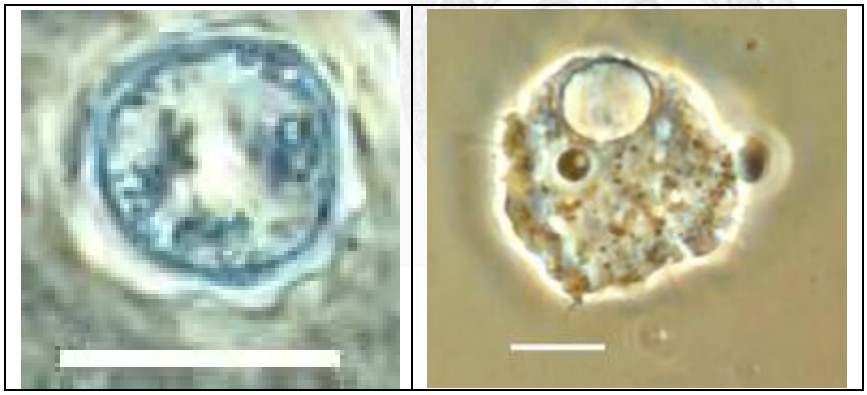


Figure 22 Trophozoite (a) and cyst (b) of *Acanthamoeba* isolate D_i1 viewed by an inverted microscope at a magnification of 1000X. (Bar = 10 µm)

(a) (b)

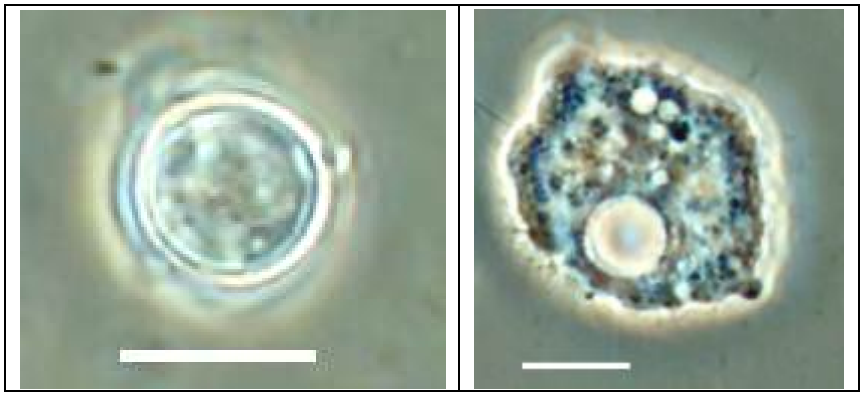


Figure 23 Trophozoite (a) and cyst (b) of *Acanthamoeba* isolate HC1_Soil_s2 viewed by an inverted microscope at a magnification of 1000X. (Bar = 10 µm)

(a) (b)

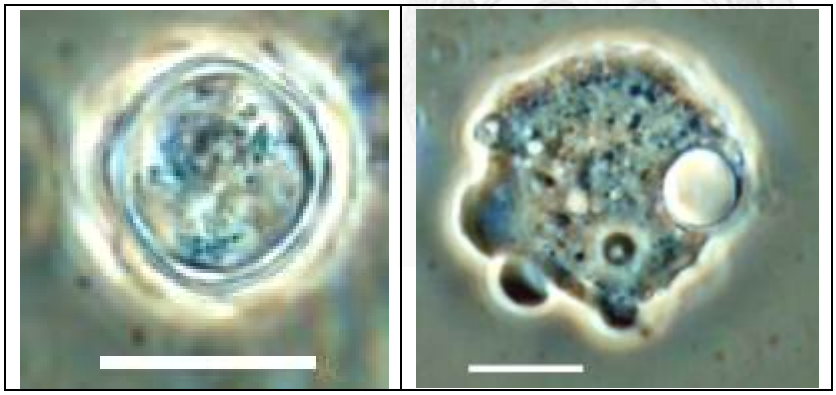


Figure 24 Trophozoite (a) and cyst (b) of *Acanthamoeba* isolate HC1_Soil_s5 viewed by an inverted microscope at a magnification of 1000X. (Bar = 10 µm)

(a) (b)

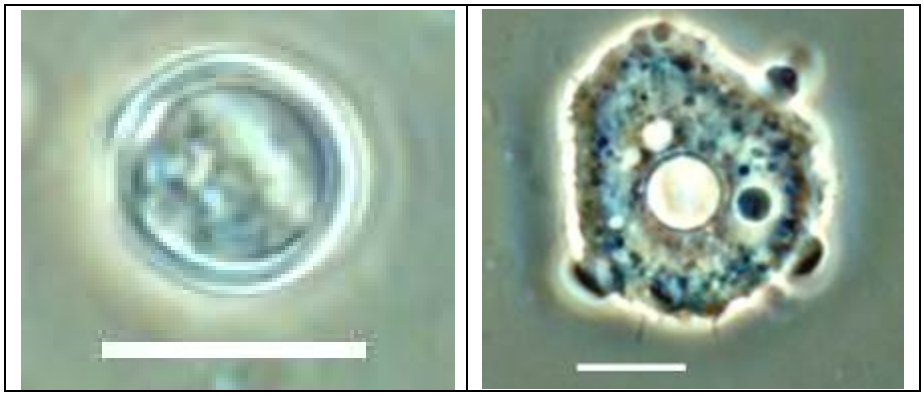


Figure 25 Trophozoite (a) and cyst (b) of *Acanthamoeba* isolate HC2_Soil_s1 viewed by an inverted microscope at a magnification of 1000X. (Bar = 10 µm)

(a) (b)

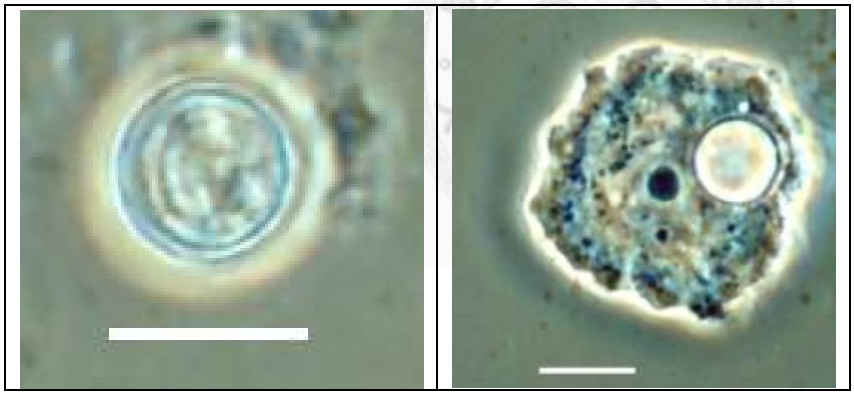


Figure 26 Trophozoite (a) and cyst (b) of *Acanthamoeba* isolate HC2_Soil_s3 viewed by an inverted microscope at a magnification of 1000X. (Bar = 10 µm)

(a) (b)

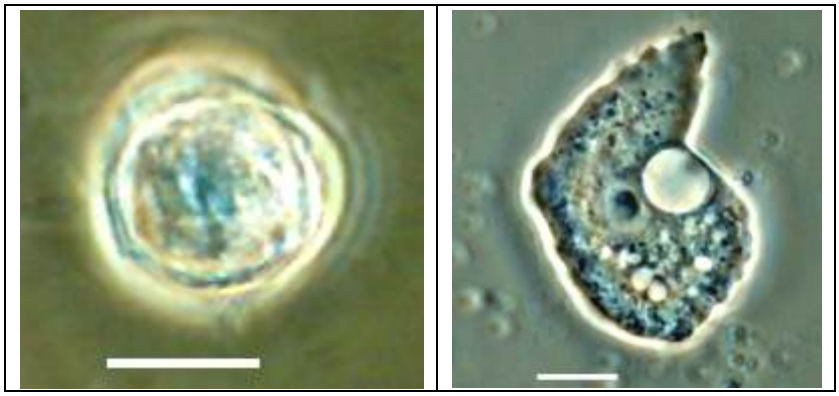


Figure 27 Trophozoite (a) and cyst (b) of *Acanthamoeba* isolate HC2_Soil_s4 viewed by an inverted microscope at a magnification of 1000X. (Bar = 10 µm)

(a) (b)

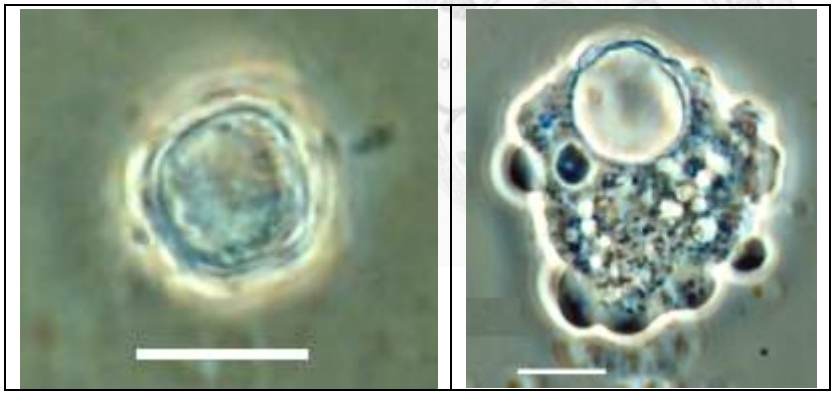


Figure 28 Trophozoite (a) and cyst (b) of *Acanthamoeba* isolate HC2_Soil_s5 viewed by an inverted microscope at a magnification of 1000X. (Bar = 10 µm)

(a) (b)

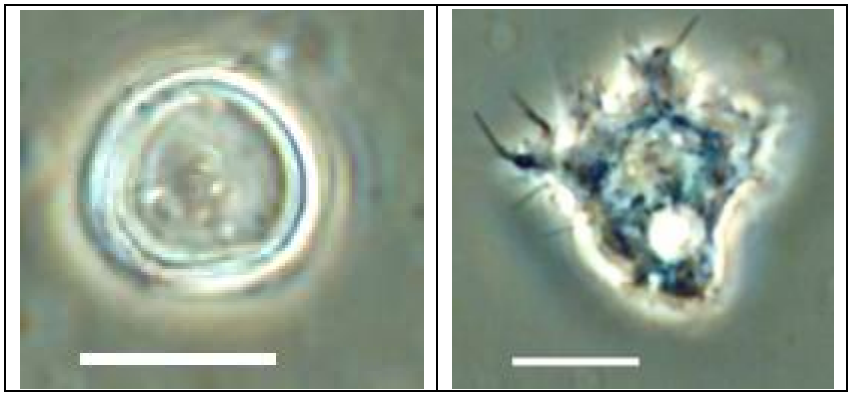


Figure 29 Trophozoite (a) and cyst (b) of *Acanthamoeba* isolate HC2_Soil_s6 viewed by an inverted microscope at a magnification of 1000X. (Bar = 10 µm)

(a) (b)

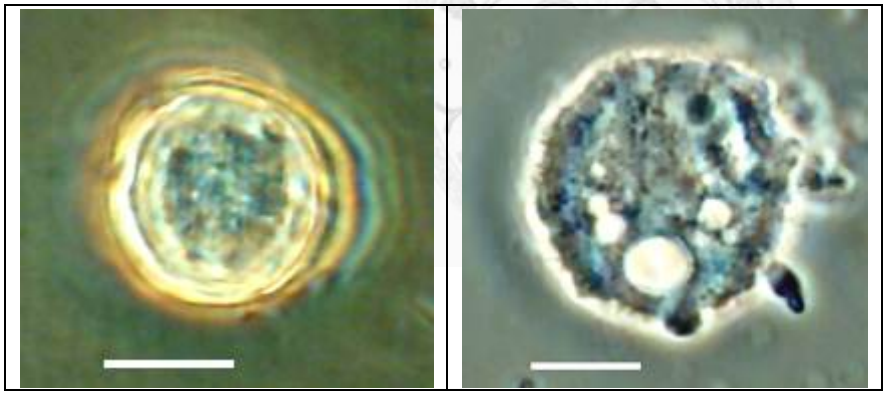


Figure 30 Trophozoite (a) and cyst (b) of *Acanthamoeba* isolate CC1_Soil_s1 viewed by an inverted microscope at a magnification of 1000X. (Bar = 10 µm)

(a) (b)

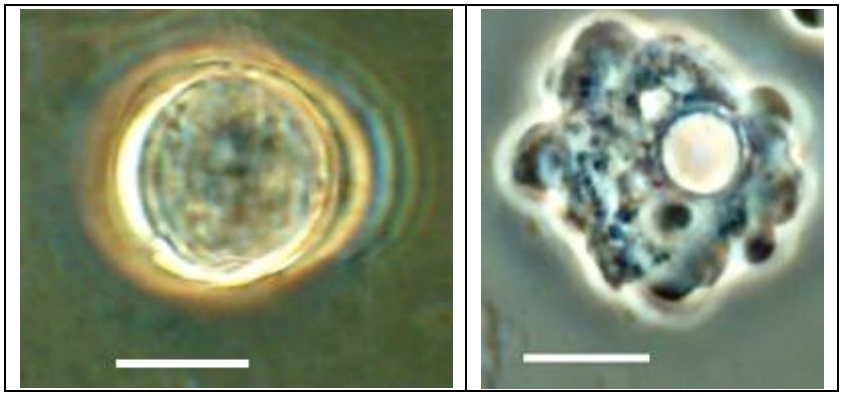


Figure 31 Trophozoite (a) and cyst (b) of *Acanthamoeba* isolate CC1_Soil_s5 viewed by an inverted microscope at a magnification of 1000X. (Bar = 10 µm)

(a) (b)

Figure 32 Trophozoite (a) and cyst (b) of *Acanthamoeba* isolate CC2_Soil_s1 viewed by an inverted microscope at a magnification of 1000X. (Bar = 10 µm)

(a) (b)

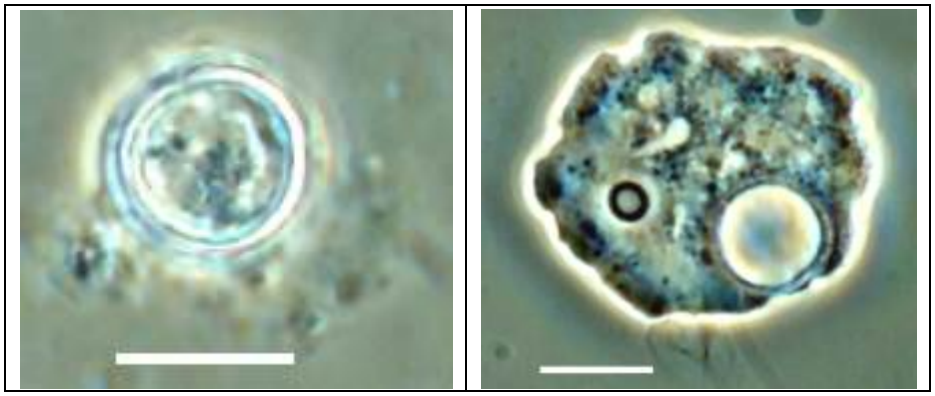


Figure 33 Trophozoite (a) and cyst (b) of *Acanthamoeba* isolate CC2_Soil_s2 viewed by an inverted microscope at a magnification of 1000X. (Bar = 10 µm)

(a) (b)

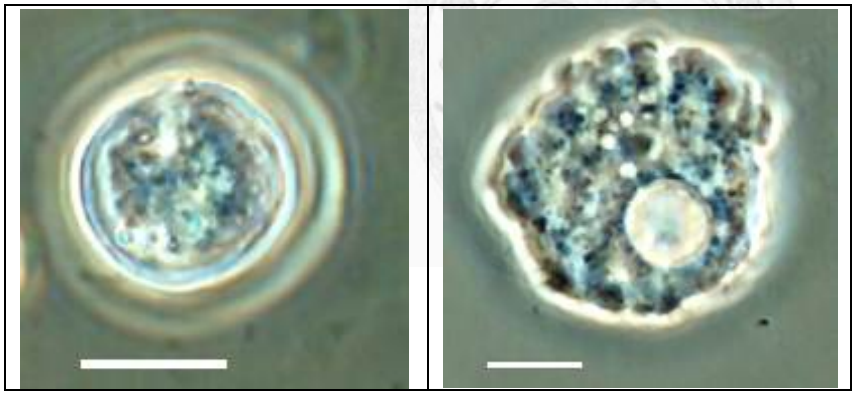


Figure 34 Trophozoite (a) and cyst (b) of *Acanthamoeba* isolate CC2_Soil_s3 viewed by an inverted microscope at a magnification of 1000X. (Bar = 10 µm)

(a) (b)

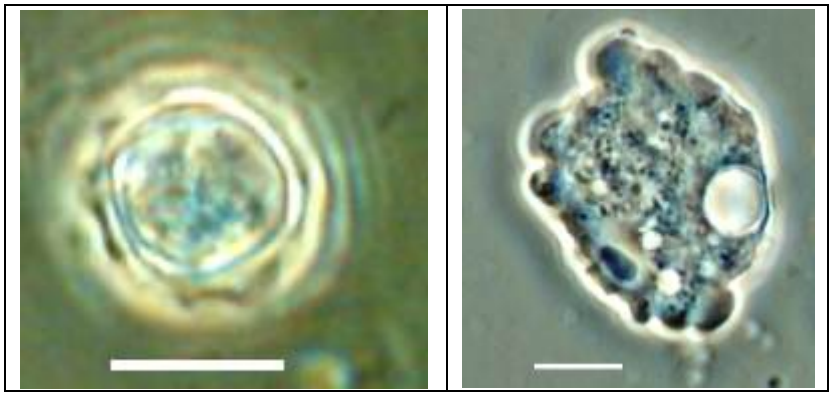


Figure 35 Trophozoite (a) and cyst (b) of *Acanthamoeba* isolate CC2_Soil_s4 viewed by an inverted microscope at a magnification of 1000X. (Bar = 10 µm)

(a) (b)

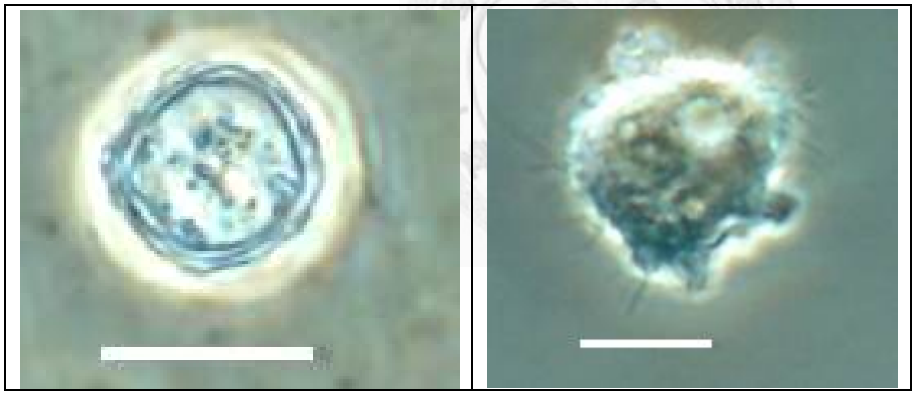


Figure 36 Trophozoite (a) and cyst (b) of *Acanthamoeba* isolate HC1_Air_s4 viewed by an inverted microscope at a magnification of 1000X. (Bar = 10 µm)

(a) (b)

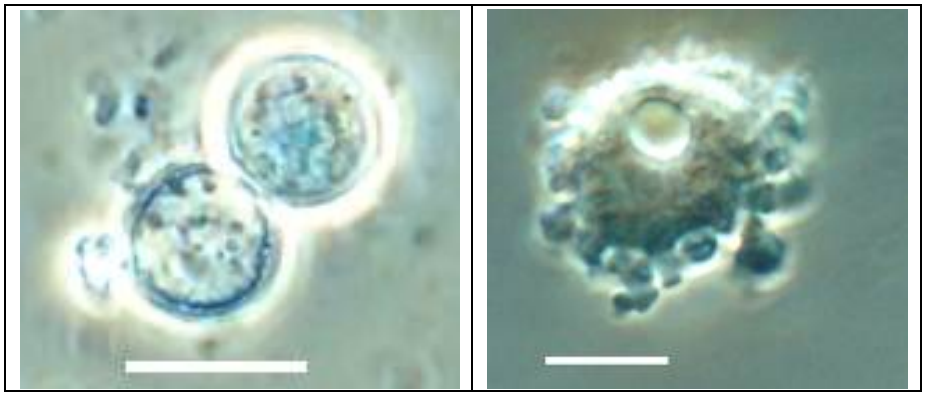


Figure 37 Trophozoite (a) and cyst (b) of *Acanthamoeba* isolate HC2_Air_s3 viewed by an inverted microscope at a magnification of 1000X. (Bar = 10 µm)

(a) (b)

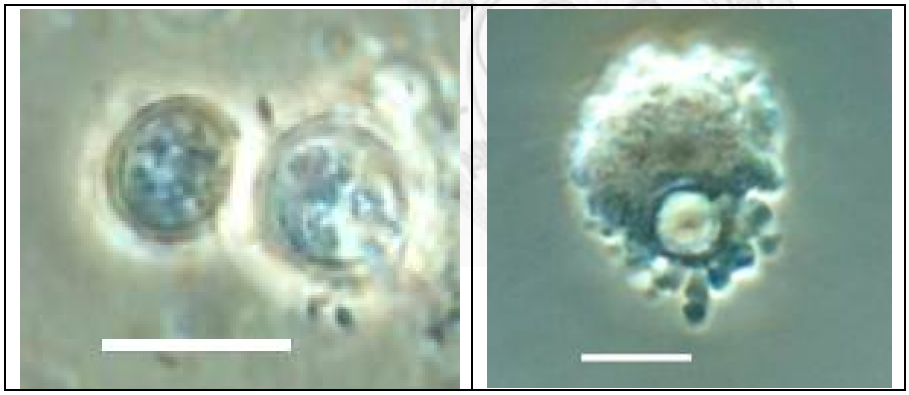


Figure 38 Trophozoite (a) and cyst (b) of *Acanthamoeba* isolate HC2_Air_s6 viewed by an inverted microscope at a magnification of 1000X. (Bar = 10 µm)

5.2.3. Gel electrophoresis of *Acanthamoeba*

The samples conducted for DNA extraction and PCR amplifications on similar time period were run on the same gel for eletrophoresis. Amplification of amoeba isolates by PCR primers JDP1

and JDP2 was successfully carried out on all 42 isolates (Fig. 40 – 44). The illustrations of amplimers ASA.S1 with a size approximately of 500 bp were observed for all isolates.

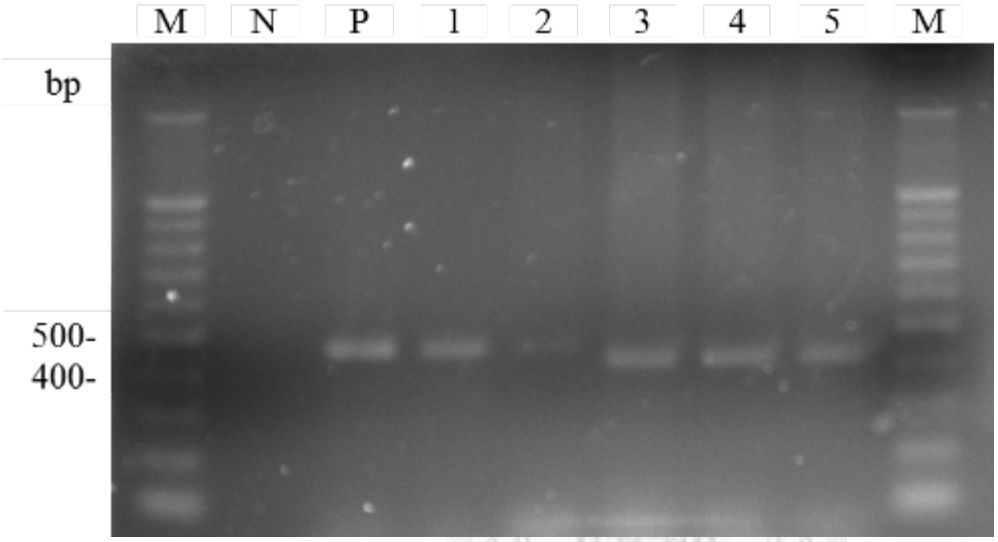


Figure 39 Gel electrophoresis of primers JDP1 and JDP2 for five isolates from air and soil samples collected on November, 2011. Lane 1: air sample from Hengchun, farmland 4 (HC1_Air_s4), lane 2: soil sample from Hengchun, farmland 2 (HC1_Soil_s2), lane 3: soil sample from Hengchun, farmland 5 (HC1_Soil_s5), lane 4: soil sample from Checheng, farmland 1 (CC1_Soil_s1), lane 5: soil sample from Checheng, farmland 5 (CC1_Soil_s5), M: size markers, lane N: negative control (ddH_2O), lane P: positive control (*Acanthamoeba polyphaga*).

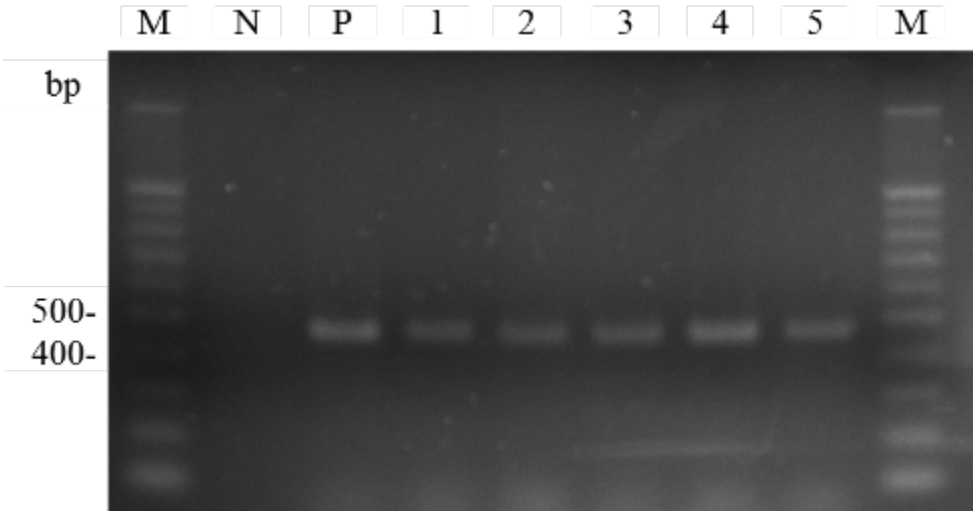


Figure 40 Gel electrophoresis of primers JDP1 and JDP2 for five isolates from water samples collected on July, 2011. Lane 1 to Lane 5 was water sample from swimming pool A. Lane1: isolate 4 (A_ i4), lane 2: isolate 5 (A_ i5), lane 3: isolate 6 (A_ i6), lane 4: isolate 7 (A_ i7), lane 5: isolate 5 (A_ i8), M: size markers, lane N: negative control (ddH_2O), lane P: positive control (*Acanthamoeba polyphaga*).

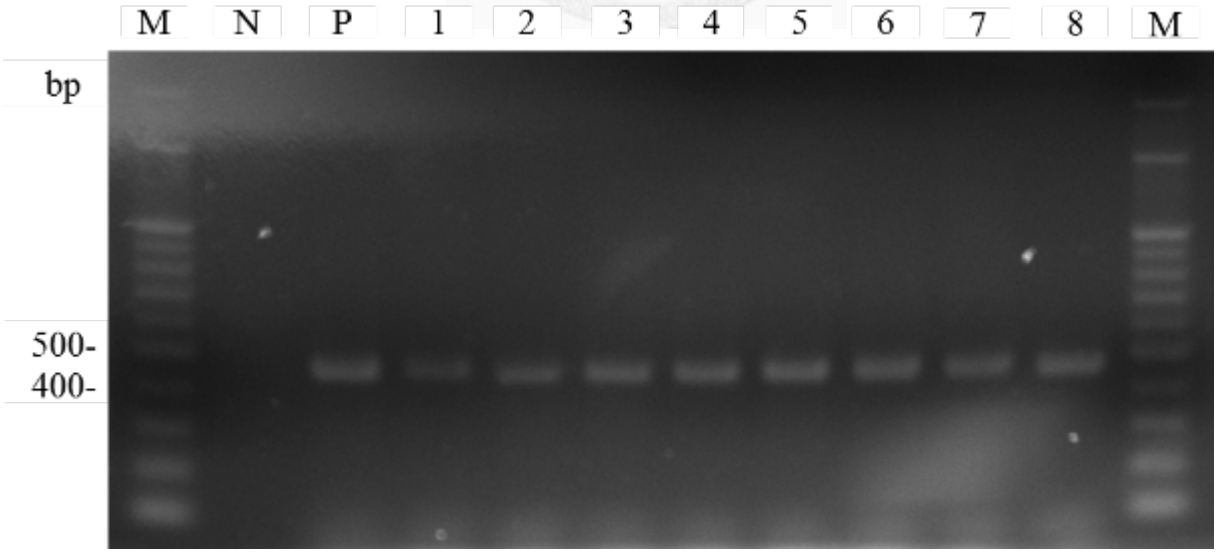


Figure 41 Gel electrophoresis of primers JDP1 and JDP2 for five isolates from water samples

collected on July, 2011. Lane 1 to Lane 8 was water sample from swimming pool E. Lane1: isolated 1 (E_i1), lane 2: isolate 2(E_i2), lane 3: isolate 3 (E_i3), lane 4: isolated 4 (E_i4), lane 5: isolate 5 (E_i5), lane 6: isolate 6 (E_i6), lane 7: isolate 7 (E_i7), lane 8: isolate 8 (E_i8), M: size markers, lane N: negative control (ddH_2O), lane P: positive control (*Acanthamoeba polyphaga*).

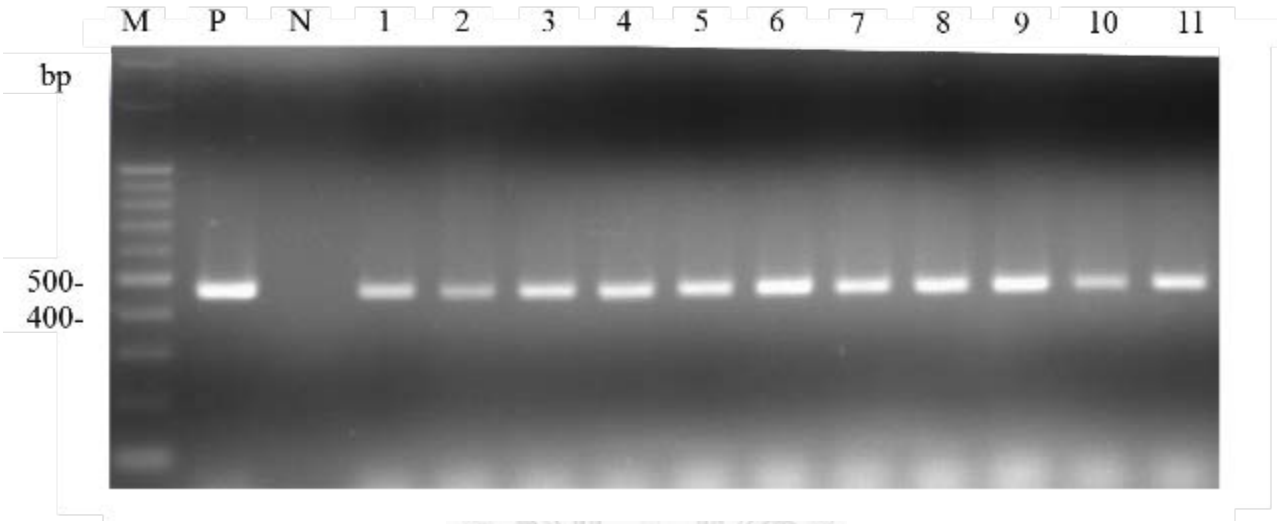


Figure 42 Gel electrophoresis of primers JDP1 and JDP2 for five isolates from air and soil samples collected on March, 2012. Lane 1: air sample from Hengchun, farmland 3 (HC2_Air_s3), lane 2: air sample from Hengchun, farmland 6 (HC2_Air_s6), lane 3: soil sample from Hengchun, farmland 1 (HC2_Soil_s1), lane 4: soil sample from Hengchun, farmland 3 (HC2_Soil_s3), lane 5: soil sample from Hengchun, farmland 4 (HC2_Soil_s4), lane 6: soil sample from Hengchun, farmland 5 (HC2_Soil_s5), lane 7: soil sample from Hengchun , farmland 6 (HC2_Soil_s6), lane 8: soil sample from Checheng, farmland 1 (CC2_Soil_s1), lane 9: soil sample from Checheng, farmland 2 (CC2_Soil_s2) , lane 10: soil sample from Checheng, farmland 3 (CC2_Soil_s3) , lane 11: soil sample from Checheng, farmland 4 (CC2_Soil_s4), M: size markers, lane N: negative control (ddH_2O), lane P: positive control (*Acanthamoeba polyphaga*).

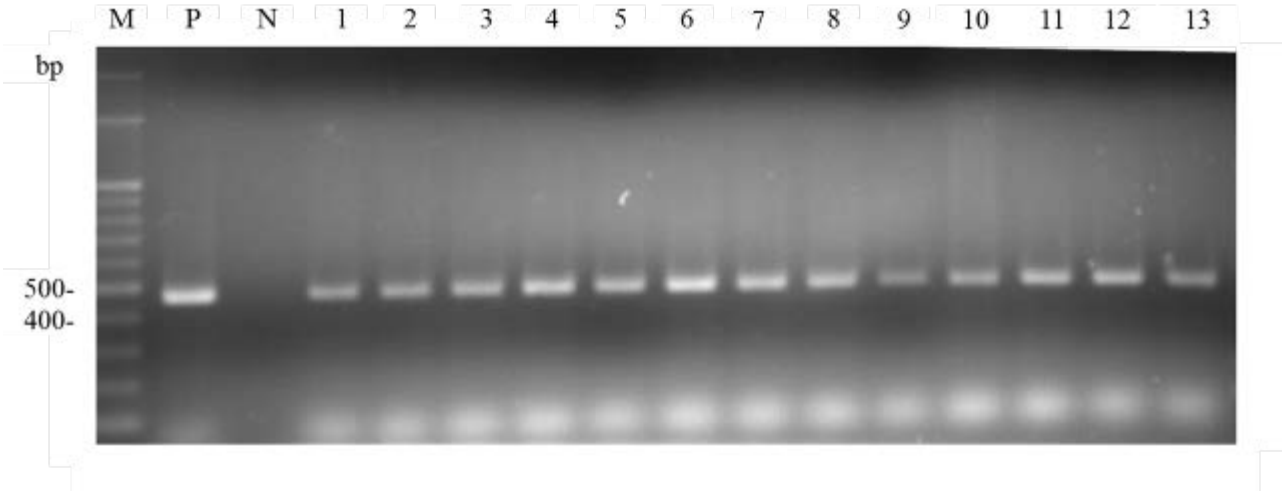


Figure 43 Gel electrophoresis of primers JDP1 and JDP2 for five isolates from water samples collected on March, 2012. Lane 1 to Lane 3 were water sample from swimming pool F, Lane 4 to Lane 8 were water sample from swimming pool B, Lane 9 to Lane 11 were water sample from swimming pool C, Lane 12 to Lane 13 were water sample from swimming pool D. Lane1: isolated 1 (F_i1), lane 2: isolate 2(F_i2), lane 3: isolate 3 (F_i1), lane 4: isolated 1 (B_i1), lane 5: isolate 2 (B_i2), lane 6: isolate 3 (B_i3), lane 7: isolate 4 (B_i4), lane 8: isolate 5 (B_i5), lane 9: isolate 1 (C_i1), lane 10: isolate 2 (C_i2), lane 11: isolate 3 (C_i3), lane 12: isolate 1 (D_i1), lane 13: isolate 2 (D_i2), M: size markers, lane N: negative control (ddH_2O), lane P: positive control (*Acanthamoeba polyphaga*).

5.2.4. DNA sequence and species identification of isolated *Acanthamoeba*

The DNA sequences of 42 isolates were compared with the BLAST in NCBI in order to identify their species. The lowest value in E Value ($E < 0.05$), the highest value in max score, total score, query coverage and max ident were taken into account. The result is presented in Table 16.

Taking the isolated A_i4 as an example, Table 16 indicate the corresponding alignment was AY026243.1(assession number) with a description of *Acanthamoeba polyphaga* strain ATCC30461 small subunit ribosomal RNA gene, complete sequence, with the lowest in E Value (E = 0), and the highest values in max score (747), total score (747), query coverage (99%) and max ident (99%). Therefore, *A. polyphaga* was considered as the species for isolate NH_i4.

Overall, the 42 isolates were groups into three species, i.e. *A. polyphaga*, *Acanthamoeba lenticulata* and *Acanthamoeba jacobsi* (Table 15). All isolates from swimming pools are all belonged to *A. polyphaga*, sharing a similarity with query coverage between 96% to 100% (Table 15). As for 13 isolates from soil, six isolates (HC1_Soil_s2, HC2_Soil_s1, HC2_Soil_s3, HC2_Soil_s6, CC2_Soil_s1 and CC2_Soil_s2) belonged to *A. polyphaga*, sharing a similarity with query coverage of 97%, and seven isolates (HC1_Soil_s5, HC2_Soil_s4, HC2_Soil_s5, CC1_Soil_s1, CC1_Soil_s5, CC2_Soil_s3 and CC2_Soil_s4) belonged to *A. lenticulata*, sharing a similarity with query coverage between 96% to 98% (Table 15). In terms of three isolates from air samples, two isolates (HC1_Air_s4 and HC2_Air_s6) belonged to *A.polyphaga*, sharing a similarity with query coverage between 96% and 97%. However, the other isolate (HC2_Air_s3) are identify as *A. jacobsi* with query coverage of 99% (Table 15).

Table 15 Sequence regions taken for genotyping and species identifications of *Acanthamoeba* isolates

No.	Sample type	*Acanthamoeba* isolate ID	Length (bp)	Regions taken for comparison	*Acanthamoeba* species	Query coverage
1	Water	A_i4	430	8 – 427	*Acanthamoeba polyphaga*	99%
2	Water	A_i5	426	8 – 425	*Acanthamoeba polyphaga*	96%
3	Water	A_i6	429	7 – 425	*Acanthamoeba polyphaga*	97%
4	Water	A_i7	429	8 – 425	*Acanthamoeba polyphaga*	97%
5	Water	A_i8	430	8 – 426	*Acanthamoeba polyphaga*	96%
6	Water	E_i1	428	6 – 425	*Acanthamoeba polyphaga*	97%
7	Water	E_i2	424	7 – 424	*Acanthamoeba polyphaga*	98%
8	Water	E_i3	428	8 – 427	*Acanthamoeba polyphaga*	100%
9	Water	E_i4	428	6 – 425	*Acanthamoeba polyphaga*	97%
10	Water	E_i5	429	8 – 426	*Acanthamoeba polyphaga*	97%
11	Water	E_i6	428	8 – 425	*Acanthamoeba polyphaga*	96%
12	Water	E_i7	430	8 – 426	*Acanthamoeba polyphaga*	97%

13	Water	E_i8	431	9 – 428	*Acanthamoeba polyphaga*	98%
14	Water	F_i1	430	6 – 427	*Acanthamoeba polyphaga*	97%
15	Water	F_i2	428	8 – 427	*Acanthamoeba polyphaga*	100%
16	Water	F_i3	430	8 – 427	*Acanthamoeba polyphaga*	99%
17	Water	B_i1	430	8 – 426	*Acanthamoeba polyphaga*	97%
18	Water	B_i2	429	7 – 425	*Acanthamoeba polyphaga*	97%
19	Water	B_i3	424	7 – 424	*Acanthamoeba polyphaga*	98%
20	Water	B_i4	430	8 – 426	*Acanthamoeba polyphaga*	96%
21	Water	B_i5	428	8 – 425	*Acanthamoeba polyphaga*	96%
22	Water	C_i1	429	8 – 426	*Acanthamoeba polyphaga*	97%
23	Water	C_i2	428	6 – 425	*Acanthamoeba polyphaga*	97%
24	Water	C_i3	431	9 – 428	*Acanthamoeba polyphaga*	98%
25	Water	D_i1	430	8 – 427	*Acanthamoeba polyphaga*	99%

26	Water	D_i2	430	6 – 427	*Acanthamoeba polyphaga*	97%
27	Soil	HC1_Soil_s2	433	10 – 429	*Acanthamoeba polyphaga*	97%
28	Soil	HC1_Soil_s5	398	8 – 393	*Acanthamoeba lenticulata*	98%
29	Soil	CC1_Soil_s1	397	8 – 392	*Acanthamoeba lenticulata*	96%
30	Soil	CC1_Soil_s5	397	8 – 392	*Acanthamoeba lenticulata*	96%
31	Soil	HC2_Soil_s1	430	8 – 429	*Acanthamoeba polyphaga*	97%
32	Soil	HC2_Soil_s3	433	10 – 429	*Acanthamoeba polyphaga*	97%
33	Soil	HC2_Soil_s4	396	8 – 392	*Acanthamoeba lenticulata*	97%
34	Soil	HC2_Soil_s5	397	8 – 392	*Acanthamoeba lenticulata*	96%
35	Soil	HC2_Soil_s6	430	8 – 429	*Acanthamoeba polyphaga*	97%
36	Soil	CC2_Soil_s1	431	8 – 427	*Acanthamoeba polyphaga*	99%
37	Soil	CC2_Soil_s2	429	6 – 426	*Acanthamoeba polyphaga*	97%
38	Soil	CC2_Soil_s3	397	8 – 392	*Acanthamoeba lenticulata*	96%
39	Soil	CC2_Soil_s4	398	8 – 393	*Acanthamoeba lenticulata*	98%

40	Air	HC1_Air_s4	429	8 – 425	*Acanthamoeba polyphaga*	97%
41	Air	HC2_Air_s3	454	8 – 453	*Acanthamoeba jacobsi*	99%
42	Air	HC2_Air_s6	430	7 – 426	*Acanthamoeba polyphaga*	96%

5.2.5. Phylogenetic analysis and genotyping

The results of 42 *Acanthamoeba* isolates identified by BLAST analysis in same accession and same query coverage were clustered into the same group. Altogether, 42 isolates were categorized into 19 groups in the present study: (E_i3 and F_i2); (A_i4); (E_i8 and C_i3); (E_i1, E_i4, F_i1, C_i2, D_i2, and CC2_Soil_s2); (A_i5); (F_i3, D_i1 and CC2_Soil_s1); (A_i6 and B_i2); (A_i7, E_i7, B_i1, HC1_Air_s4); (A_i8 and B_i4); (HC2_Air_s6); (E_i6 and B_i5); (E_i2 and B_i3); (E_i5 and C_i1); (HC1_Soil_s2 and HC2_Soil_s3); (HC1_Soil_s5 and CC2_Soil_s4); (HC2_Soil_s4); (CC1_Soil_s1, CC1_Soil_s5, HC2_Soil_s5, and CC2_Soil_s3); (HC2_Soil_s1 and HC2_Soil_s6); and (HC2_Air_s3) (Table 17). The 19 groups were used to conduct phylogenetic analysis (Fig. 44).

By taking isolate HC2_Air_s3 as an example, it was in the same cluster with a reference strain of T15 - *A. jacobsi* (AY262362). Therefore, the phylogenetic analysis illustrated *Acanthamoeba* HC2_Air_s3 belonged to genotype T15 as it was in the same cluster of T15 - *A. jacobsi* (AY262362) (Fig. 44). Compared to 12 reference strains (Table 9), the 19 sequence groups were divided into three genotypes, i.e. T4, T5 and T15 (Fig. 44 and Table 16). Overall, 34 out of 42 isolates belonged to T4 genotype (34/42, 80.95%), seven isolates were genotype T5 (7/42, 16.67%), and only one isolate was recognized as genotype T15 (1/42, 2.38%) (Fig. 44 and Table 16). In detail, all of the 26 isolates from swimming pools belonged to genotype T4 (26/26, 100%). As for 13 isolates from soil

samples, six isolates were genotype T4 (6/13, 46.2%) and the other seven isolates belonged to genotype T5 (7/13, 53.8%) (Fig. 44 and Table 16). For three isolates from samples, two isolates were genotype T4 (2/3, 66.6%) while the other belonged to genotype T15 (1/3, 33.3%) (Fig. 44 and Table 16).

Acanthamoeba isolates from soil and isolates from ambient air in the same sampling day and same onion farmland were revealed to have the difference in DNA base pairs. Table 17 indicated the alignment results of different isolates from the same sampling day and same sampling site. *Acanthamoeba* (HC2_Soil_s3) isolated from soil in Hengchun Township, farmland 3 and *Acanthamoeba* (HC2_Air_s3) isolated from soil in Hengchun Township, farmland 3 revealed 97% in identities and the gaps was 3%. When it comes to sampling in Hengchun Township, farmland 6, soil isolate (HC2_Soil_s6) and air isolate (HC2_Air_s6) revealed 85% in identities and the gaps was 15%.

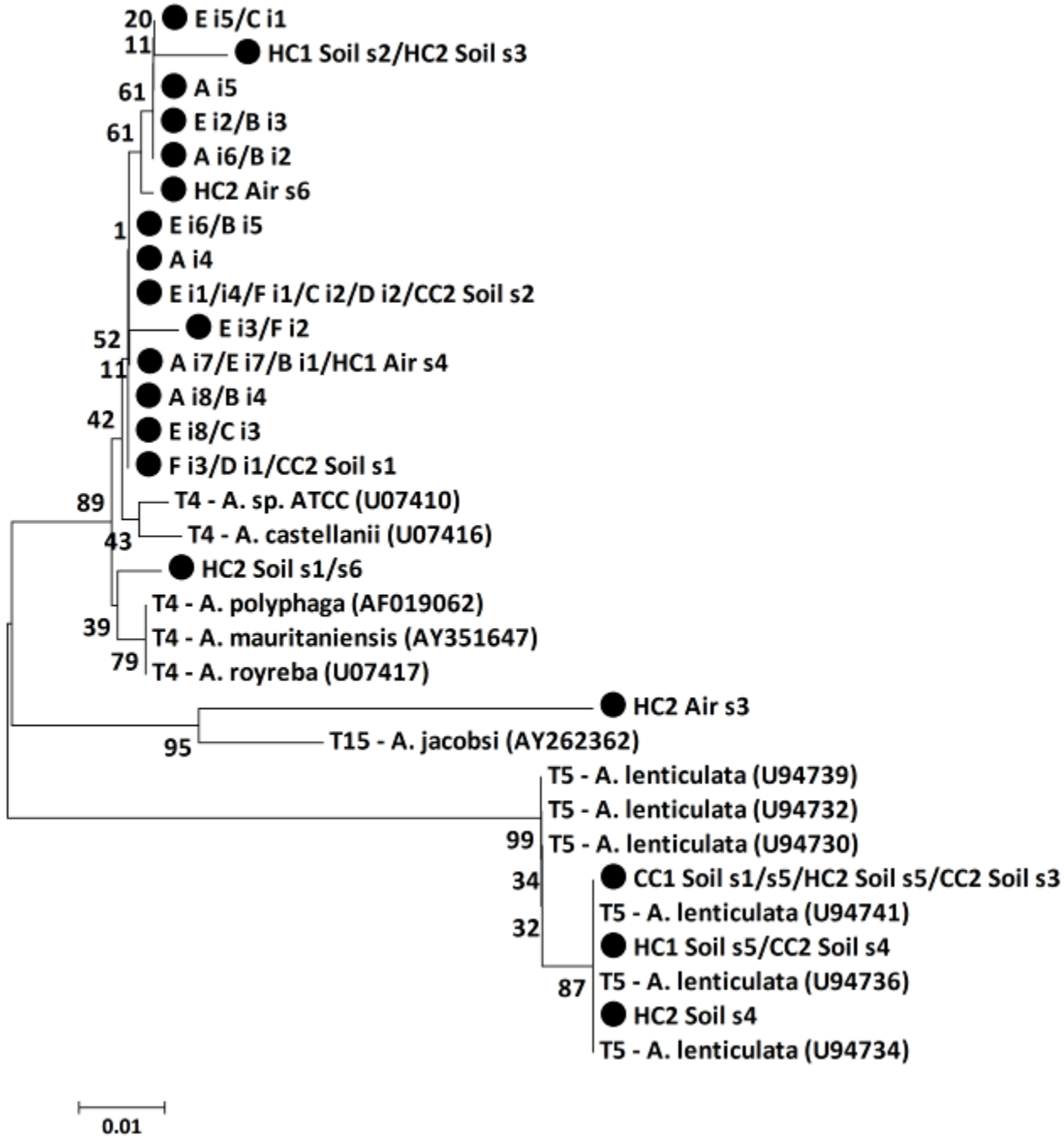


Figure 44 Neighbor-joining tree depicting the relationships between 42 isolates and 12 reference strains of *Acanthamoeba* representing genotypes T4, T5 and T15. GenBank accession numbers for reference sequences are indicated at the ends of sequence designations. Filled circles represent environmental isolates being grouped based on BLAST results. The percentages of replicate trees in

which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches.

Table 16 Summary of 42 isolates for their genotype and species

Group	Genotype	Species classification	Strain	Abbreviation[a]
1	T4	*Acanthamoeba polyphaga*	E_i3 F_i2	E i3/F i2
2	T4	*Acanthamoeba polyphaga*	A_i4	A i4
3	T4	*Acanthamoeba polyphaga*	E_i8 C_i3	E i8/C i3
4	T4	*Acanthamoeba polyphaga*	E_i1 E_i4 F_i1 C_i2 D_i2 CC2_Soil_s2	E i1/i4/F i1/C i2/D i2/CC2 Soil s2
5	T4	*Acanthamoeba polyphaga*	A_i5	A i5
6	T4	*Acanthamoeba polyphaga*	F_i3 D_i1 CC2_Soil_s1	F i3/D i1/CC2 Soil s1
7	T4	*Acanthamoeba polyphaga*	A_i6 B_i2	A i6/B i2
8	T4	*Acanthamoeba*	A_i7	NH i7/E i7/B i1/HC1 Air s4

		polyphaga	E_i7 B_i1 HC1_Air_s4	
9	T4	*Acanthamoeba polyphaga*	A_i8 B_i4	A i8/B i4
10	T4	*Acanthamoeba polyphaga*	HC2_Air_s6	HC2 Air s6
11	T4	*Acanthamoeba polyphaga*	E_i6 B_i5	E i6/B i5
12	T4	*Acanthamoeba polyphaga*	E_i2 B_i3	E i2/B i3
13	T4	*Acanthamoeba polyphaga*	E_i5 C_i1	E i5/C i1
14	T4	*Acanthamoeba polyphaga*	HC1_Soil_s2 HC2_Soil_s3	HC1 Soil s2/HC2 Soil s3
15	T4	*Acanthamoeba polyphaga*	HC2_Soil_s1 HC2_Soil_s6	HC2 Soil s1/HC2 Soil s6
16	T5	*Acanthamoeba lenticulata*	HC1_Soil_s5 CC2_Soil_s4	HC1 Soil s5/CC2 Soil s4
17	T5	*Acanthamoeba lenticulata*	CC1_Soil_s1 CC1_Soil_s5 HC2_Soil_s5 CC2_Soil_s3	CC1 Soil s1/s5/HC2 Soil s5/CC2 Soil s3
18	T5	*Acanthamoeba lenticulata*	HC2_Soil_s4	HC2 Soil s4
19	T15	*Acanthamoeba sp.*	HC2_Air_s3	HC2 Air s3

[a] Abbreviation is the combination of sample name utilized in phylgenetic analysis tree

Table 17 Alignment results of different isolates from the same sampling day and same sampling site

City	Location	Sampling site	Sample type	Sampling date (Day/Month/Year)	*Acanthamoeba* isolate ID	Length taken for comparison (bp)	Identities	Gaps
Pingtung County, Taiwan	Onion farmland	Hengchun Township, farmland 3	Soil	03/21/2012	HC2_Soil_s3	430	419/430 (97%)	11/430 (3%)
			Air	03/21/2012	HC2_Air_s3	430		
Pingtung County, Taiwan	Onion farmland	Hengchun Township, farmland 6	Soil	03/22/2012	HC2_Soil_s6	462	393/462 (85%)	69/462 (15%)
			Air	03/22/2012	HC2_Air_s6	462		

5.2.6. Thermotolerance and osmotolerance testing

Overall, there were 28 environmental isolates used for thermotolerance and osmotolerance testing (Table 18).

Table 18 Environmental isolates selected for thermotolerance and osmotolerance testing

Location	Sampling site	Sample type	Sample ID	*Acanthamoeba* isolate ID	Genotype	Thermotolerance and osmotolerance[a]
Swimming pool	Swimming pool A	water	ASP Water Sample1	A_ i4	T4	Y
Swimming pool	Swimming pool A	water	ASP Water Sample1	A_ i5	T4	N
Swimming pool	Swimming pool A	water	ASP Water Sample2	A_ i6	T4	Y
Swimming pool	Swimming pool A	water	ASP Water Sample2	A_ i7	T4	N
Swimming pool	Swimming pool A	water	ASP Water Sample2	A_ i8	T4	N
Swimming pool	Swimming pool E	water	ESP Water Sample1	E_i1	T4	N
Swimming pool	Swimming pool E	water	ESP Water Sample1	E_i2	T4	Y
Swimming pool	Swimming pool E	water	ESP Water Sample1	E_i3	T4	N
Swimming pool	Swimming pool E	water	ESP Water Sample2	E_i4	T4	N
Swimming pool	Swimming pool E	water	ESP Water Sample2	E_i5	T4	N
Swimming pool	Swimming pool E	water	ESP Water Sample2	E_i6	T4	N
Swimming pool	Swimming pool E	water	ESP Water Sample2	E_i7	T4	N
Swimming pool	Swimming pool E	water	ESP Water Sample2	E_i8	T4	Y

Swimming pool	Swimming pool F	water	FSP Water Sample1	F_i1	T4	Y
Swimming pool	Swimming pool F	water	FSP Water Sample1	F_i2	T4	N
Swimming pool	Swimming pool F	water	FSP Water Sample2	F_i3	T4	Y
Swimming pool	Swimming pool B	water	BSP Water Sample1	B_i1	T4	Y
Swimming pool	Swimming pool B	water	BSP Water Sample1	B_i2	T4	N
Swimming pool	Swimming pool B	water	BSP Water Sample2	B_i3	T4	Y
Swimming pool	Swimming pool B	water	BSP Water Sample2	B_i4	T4	N
Swimming pool	Swimming pool B	water	BSP Water Sample2	B_i5	T4	N
Swimming pool	Swimming pool C	water	CSP Water Sample1	C_i1	T4	Y
Swimming pool	Swimming pool C	water	CSP Water Sample1	C_i2	T4	N
Swimming pool	Swimming pool C	water	CSP Water Sample2	C_i3	T4	Y
Swimming pool	Swimming pool D	water	DSP Water Sample1	D_i1	T4	Y
Swimming pool	Swimming pool D	water	DSP Water Sample2	D_i2	T4	Y
Onion farmland	Hengchun Township, farmland 2	Soil	HC1FARM Soil Sample2	HC1_Soil_s2	T4	Y
Onion farmland	Hengchun Township, farmland 5	Soil	HC1FARM Soil Sample5	HC1_Soil_s5	T4	Y
Onion farmland	Hengchun Township, farmland 1	Soil	HC2FARM Soil Sample1	HC2_Soil_s1	T4	Y
Onion farmland	Hengchun Township, farmland 2	Soil	HC2FARM Soil Sample2	Sample loss[b]	NA[c]	NA
Onion farmland	Hengchun Township, farmland 3	Soil	HC2FARM Soil Sample3	HC2_Soil_s3	T4	Y

Onion farmland	Hengchun Township, farmland 4	Soil	HC2FARM Soil Sample4	HC2_Soil_s4	T4	Y
Onion farmland	Hengchun Township, farmland 5	Soil	HC2FARM Soil Sample5	HC2_Soil_s5	T5	Y
Onion farmland	Hengchun Township, farmland 6	Soil	HC2FARM Soil Sample6	HC2_Soil_s6	T4	Y
Onion farmland	Checheng Township, farmland 1	Soil	CC1FARM Soil Sample1	CC1_Soil_s1	T5	Y
Onion farmland	Checheng Township, farmland 5	Soil	CC1FARM Soil Sample5	CC1_Soil_s5	T5	Y
Onion farmland	Checheng Township, farmland 1	Soil	CC2FARM Soil Sample1	CC2_Soil_s1	T4	Y
Onion farmland	Checheng Township, farmland 2	Soil	CC2FARM Soil Sample2	CC2_Soil_s2	T4	Y
Onion farmland	Checheng Township, farmland 3	Soil	CC2FARM Soil Sample3	CC2_Soil_s3	T5	Y
Onion farmland	Checheng Township, farmland 4	Soil	CC2FARM Soil Sample4	CC2_Soil_s4	T4	Y
Onion farmland	Hengchun Township, farmland 2	Air	HC1FARM Air Sample2	ND[d]	ND	ND
Onion farmland	Hengchun Township, farmland 4	Air	HC1FARM Air Sample4	HC1_Air_s4	T4	Y
Onion farmland	Hengchun Township, farmland 1	Air	HC2FARM Air Sample1	ND	ND	ND
Onion farmland	Hengchun Township,	Air	HC2FARM Air Sample2	ND	ND	ND

	farmland 2					
Onion farmland	Hengchun Township, farmland 3	Air	HC2FARM Air Sample3	HC2_Air_s3	T15	Y
Onion farmland	Hengchun Township, farmland 4	Air	HC2FARM Air Sample4	ND	ND	ND
Onion farmland	Hengchun Township, farmland 5	Air	HC2FARM Air Sample5	ND	ND	ND
Onion farmland	Hengchun Township, farmland 6	Air	HC2FARM Air Sample6	HC2_Air_s6	T4	Y
Onion farmland	Checheng Township, farmland 1	Air	CC1FARM Air Sample1	ND	ND	ND
Onion farmland	Checheng Township, farmland 5	Air	CC1FARM Air Sample5	ND	ND	ND
Onion farmland	Checheng Township, farmland 1	Air	CC2FARM Air Sample1	ND	ND	ND
Onion farmland	Checheng Township, farmland 2	Air	CC2FARM Air Sample2	ND	ND	ND
Onion farmland	Checheng Township, farmland 3	Air	CC2FARM Air Sample3	ND	ND	ND
Onion farmland	Checheng Township, farmland 4	Air	CC2FARM Air Sample4	ND	ND	ND

a Y: isolates were tested for thermotolerance and osmotolerance, N: isolates were not used for thermotolerance and osmotolerance testing

b Sample loss during transportation

c NA: not available

d ND: non-detectable

The results of thermotolerance and osmotolerance assays in the present study are summarized in Table 19 and Table 20. All 28 isolates could grow at 30°C, number of *Acanthamoeba* per microscopic field ranged from 122 to 316 (Table 19). For 12 isolates of swimming pools, only one isolate from A swimming pool could not grow under 37°C, and all 12 isolates could not grow at 42°C or 52°C. Numbers of *Acanthamoeba* per microscopic field in 12 isolates and ATCC reference strains are higher at 30°C than 37°C (Fig. 45). Numbers of *Acanthamoeba* per microscopic field in 12 isolates are all higher than ATCC reference strains when incubated at 30°C (Fig. 45). Except for one isolate (A_i4), rest 11 isolates can grew and six isolates were higher than ATCC reference strains when incubated at 37°C (Fig. 45). In terms of osmotolerance, all 12 isolates tolerated at 0.5 M mannitol with number of *Acanthamoeba* per microscopic field 18 to 100 and tolerated at 1 M mannitol with number of *Acanthamoeba* per microscopic field ranged from 2 to 93 (Table 19). The highest numbers of *Acanthamoeba* per microscopic field in 12 isolates are without mannitol, followed by 0.5 M mannitol and the lowest is 1 M mannitol (Fig. 46). The numbers of *Acanthamoeba* per microscopic field are higher than reference strains in only two isolates (E_i2 and B_i3) when incubated at 1 M mannitol (Fig. 46).

As for 13 isolates from soil, seven isolates tolerated up to 52°C with number of *Acanthamoeba* per microscopic field ranged from 27 to 101, nine isolates survived and grow under 42°C with number of *Acanthamoeba* per microscopic field ranged from 31 to 138, and all isolates grew at

37°C with number of *Acanthamoeba* per microscopic field ranged from 34 to 455. The numbers of *Acanthamoeba* per microscopic field in five out of 13 isolates (38.46%) are higher when incubated at 37°C than 30°C (Fig. 47 and Table 19). The numbers of *Acanthamoeba* per microscopic field are higher than reference strains in 10 isolates when incubated at 37°C (Fig. 47 and Table 19). Thers were nine isolates survived and grow under 42°C. But only seven isolates retained their viability after 24 hours exposure 52◦C and migrated at 30◦C.When testing for osmotolerance, only one isolate (HC2_Soil_s6) survived at 1 M mannitol with 4 *Acanthamoeba* per microscopic field, but all 13 isolates grew at 0.5 M mannitol with number of *Acanthamoeba* per microscopic field ranged from 22 to 140 (Table 19). The highest numbers of *Acanthamoeba* per microscopic field in 13 isolates are without mannitol, followed by 0.5 M mannitol and the lowest is 1 M mannitol (Fig. 48 and Table 19). The numbers of *Acanthamoeba* per microscopic field in 13 isolates are all lower than ATCC reference strains when incubated at 1 M mannitol (Fig. 48 and Table 19).

In terms of three isolates from air samples, two isolates can grew at 37°C with number of *Acanthamoeba* per microscopic field 94 and 156, however, none of the three isolates was observed under 42°C or 52°C. The numbers of *Acanthamoeba* per microscopic field in two out of three isolates (66.67%) are higher when incubated at 37°C than 30°C (Fig. 49 and Table 19). The numbers of *Acanthamoeba* per microscopic field are higher than ATCC reference strains in two isolates (HC2_Air_s3 and HC2_Air_s6) when incubated at 37°C (Fig. 49 and Table 19). As for

osmotolerance testing, only one isolate (HC1_Air_s4) grew under 0.5 M mannitol with 38 *Acanthamoeba* per microscopic field and 1 M for mannitol with two *Acanthamoeba* per microscopic field, while another isolate (HC2_Air_s6) could only grew at 0.5 M mannitol with 52 *Acanthamoeba* per microscopic field (Table 19). The highest numbers of *Acanthamoeba* per microscopic field in three isolates are without mannitol, followed by 0.5 M mannitol and the lowest is 1 M mannitol (Fig. 50 and Table 19). The numbers of *Acanthamoeba* per microscopic field in three isolates are all lower than ATCC reference strains when incubated at 1 M mannitol (Fig. 50 and Table 19).

Table 19 Results of genotype, thermotolerance and osmotolerance testing shown by number of *Acanthamoeba* for water, soil and air samples and two *Acanthamoeba* reference strains

No.	*Acanthamoeba* isolate ID	Genotype	Mean number ± SD of *Acanthamoeba* per microscopic field[a]					
			At 30°C, without mannitol, 10 days	At 37°C, 10 days	At 42°C, 10 days	At 52°C (24 hours), followed by 30°C, 9 days	At 30°C, with 0.5 M mannitol, 10 days	At 30°C, with 1 M mannitol, 10 days
ENVIRONMENTAL ISOLATES								
1	A_i4	T4	122.04±24.16	0	0	0	17.85±10.40	8.41±6.53
2	A_i6	T4	148.33±28.19	115.81±26.18	0	0	69.33±19.42	5.56±3.64
3	E_i2	T4	130.48±22.94	38.26±40.57	0	0	99.85±38.54	87.93±18.71
4	E_i8	T4	290.74±16.34	46.41±27.44	0	0	66.52±23.04	1.66±2.63
5	F_i1	T4	178.71±31.72	38.11±15.82	0	0	54.70±20.51	2.67±2.39
6	F_i3	T4	187.48±38.2	126.3±25.33	0	0	96.33±30.93	19.11±10.17

7	B_i1	T4	176.74±28.11	134.37±28.39	0	0	97±24.21	20.19±10.78
8	B_i3	T4	153.15±25.26	62.92±25.53	0	0	116.78±32.23	93.19±20.47
9	C_i1	T4	162.11±46.86	120.96±25.27	0	0	86.59±29.65	28.70±17.66
10	C_i3	T4	316.19±38.5	70.48±24.45	0	0	86.04±25.42	3.44±4.03
11	D_i1	T4	164.37±35.43	95.74±13.21	0	0	64.26±25.4	9.37±8.35
12	D_i2	T4	157.89±38.92	26.19±10.02	0	0	43.19±22.07	2.26±3.02
13	HC1_Soil_s2	T4	384.63±31.39	454.81±39.45	0	0	140.3±46.81	0
14	HC1_Soil_s5	T4	162.70±22.18	318.33±135.55	113.56±23.63	100.52±17.45	42.19±10.28	0
15	HC2_Soil_s1	T4	124.52±21.81	63.81±18.05	0	0	102.22±33.84	0

16	HC2_Soil_s3	T4	297.52±34.04	300.85±43.04	0	0	124.22±31.96	0
17	HC2_Soil_s4	T4	60.04±12.04	34.33±15.51	30.37±12.68	27.37±13.69	47.37±17.92	0
18	HC2_Soil_s5	T5	264.3±45.31	157.48±23.35	96.3±19.8	42.37±14.7	52.85±18.53	0
19	HC2_Soil_s6	T4	104.44±23.44	45.3±11.51	0	0	88.15±22.17	4.22±12.25
20	CC1_Soil_s1	T5	335.56±64.87	133.52±32.24	122.07±18.01	85.89±15.99	69.81±30.28	0
21	CC1_Soil_s5	T5	191.78±15.91	192.70±19.43	49.26±22.72	37.07±11.31	21.96±11.4	0
22	CC2_Soil_s1	T4	238.26±34.51	226.93±27.85	138.44±26.78	0	52.52±15.62	0
23	CC2_Soil_s2	T4	211.11±26.9	196.19±39.36	101.59±23.54	0	41.85±18.72	0
24	CC2_Soil_s3	T5	254.22±41.53	163.48±47.45	98.63±36.18	52.81±18.15	58±25.08	0
25	CC2_Soil_s4	T4	141.92±31.5	274.52±41.33	91±25.84	78.22±25.69	35.22±16.04	0

26	HC1_Air_s4	T4	195.78±36	0	0	0	38.15±16.77	2.3±1.71
27	HC2_Air_s3	T15	76.44±19.37	93.96±13.17	0	0	0	0
28	HC2_Air_s6	T4	58.70±15.05	155.59±35.66	0	0	52.33±11.63	0
REFERENCE STRAINS								
1	*Acanthamoeba polyphaga (*ATCC 30461) from *Acanthamoeba* keratitis[b]		76.04 ± 48.29	63.85 ± 68.18	0	0	77.37 ± 14.71	78.07 ± 41.36
2	*Acanthamoeba castellanii* (ATCC 30234) from Yeast culture[c]		45.04 ± 15.01	29.19 ± 8.15	0	0	15.41 ± 7.03	5.78 ± 6.36

a Mean number of *Acanthamoeba* (n=3)

b *Acanthamoeba polyphaga* (ATCC 30461), isolated from *Acanthamoeba* keratitis, genotype T4 (Thompson et al., 2008), was used as a reference of potentially pathogenic isolate

c *Acanthamoeba castellanii* (ATCC 30234), isolated from yeast culture, was used as a reference examples of probably pathogenic isolates (Maghsood et al., 2005)

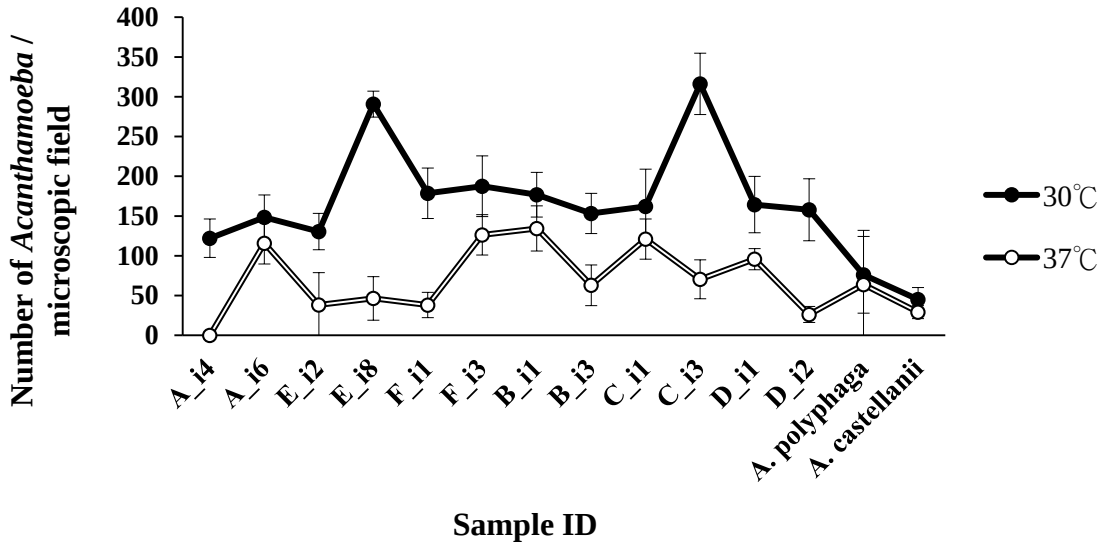


Figure 45 Results of thermotolerance testing for reference strains and 12 *Acanthamoeba* isolates collected from water of swimming pools (n=3). There were no *Acanthamoeba* including isolates and reference strains incubated at 42^{o}C and 52^{o}C

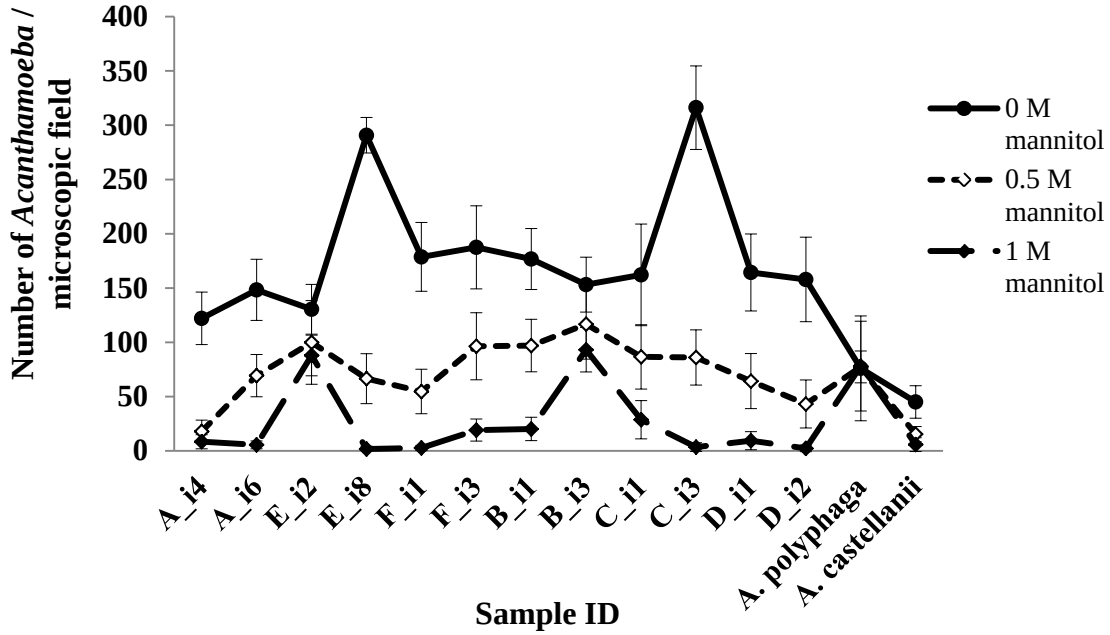


Figure 46 Results of osmotolerance testing for reference strains and 12 *Acanthamoeba* isolates

collected from water of swimming pools (n=3).

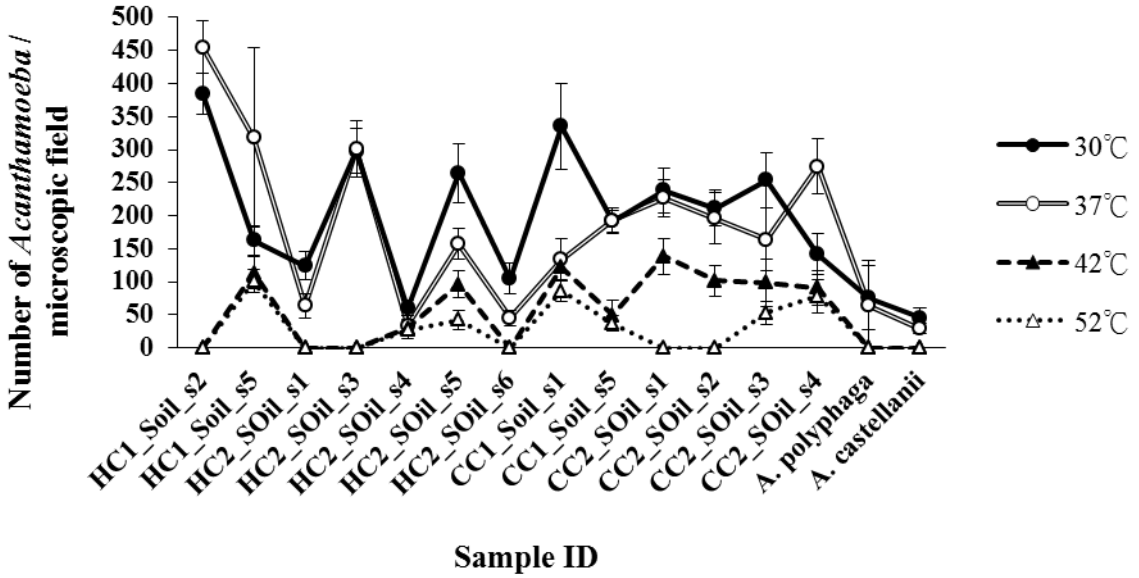


Figure 47 Results of thermotolerance testing for reference strains and 13 *Acanthamoeba* isolates collected from soil of onion farmlands (n=3)

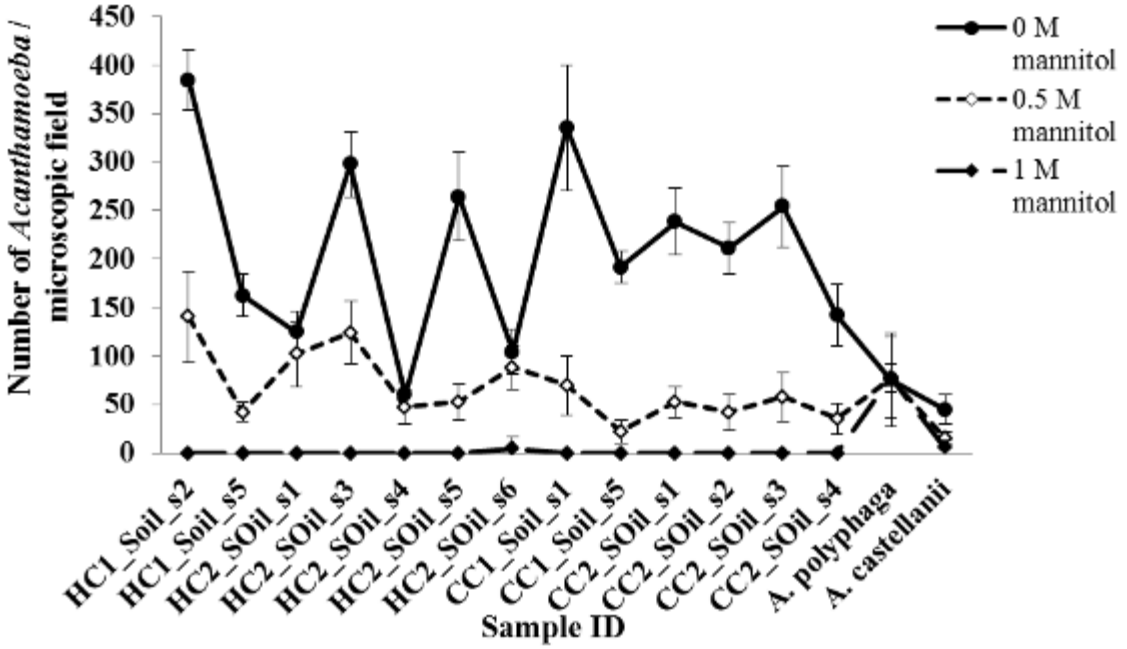


Figure 48 Results of osmotolerance testing for reference strains and 13 *Acanthamoeba* isolates collected from soil of onion farmlands (n=3).

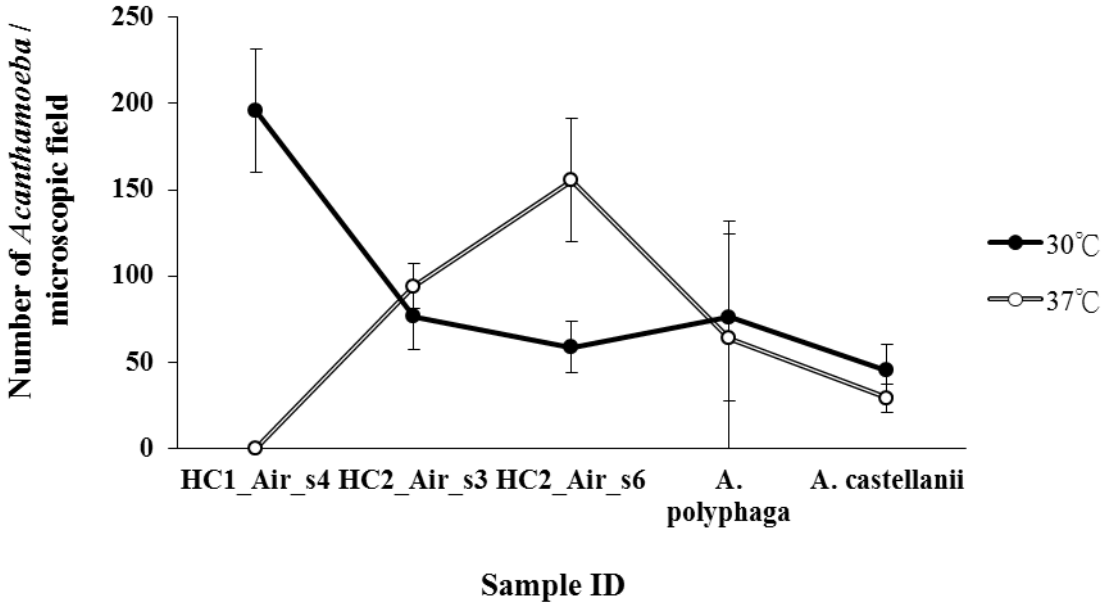


Figure 49 Results of thermotolerance testing for reference strains and 3 *Acanthamoeba* isolates collected from air of onion farmlands (n=3). There were no *Acanthamoeba* including isolates and

reference strains incubated at 42°C and 52°C

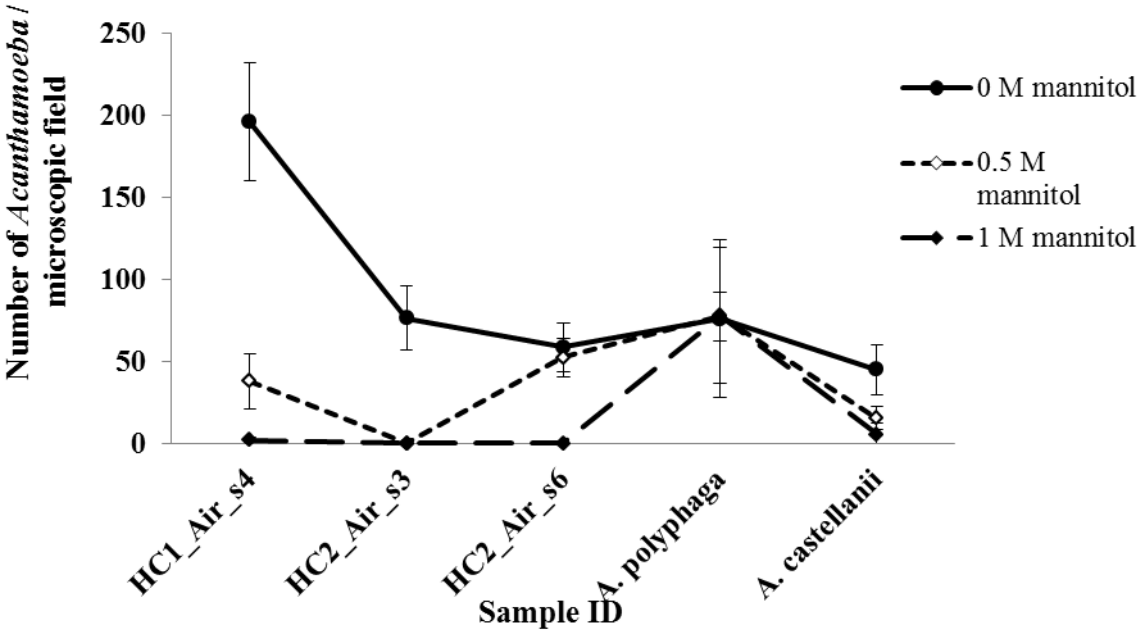


Figure 50 Results of osmotolerance testing for reference strains and 3 *Acanthamoeba* isolates collected from air of onion farmlands (n=3).

Table 20 Results of genotypes, thermotolerance and osmotolerance testing shown by semi-quantitative score value for environmental samples and reference strains

No.	*Acanthamoeba* isolate ID	Sampling site	Sample type	Genotype	Score[a]					
					At 30°C, without mannitol, 10 days	At 37°C, 10 days	At 42°C, 10 days	At 52°C (24 hours), followed by 30°C, 9 days	At 30°C, with 0.5 M mannitol, 10 days	At 30°C, with 1 M mannitol, 10 days
ENVIRONMENTAL ISOLATES										
1	A_i4	Swimming pool A	Water	T4	5+	—	—	—	2+	+
2	A_i6	Swimming pool A	Water	T4	5+	5+	—	—	4+	+
3	E_i2	Swimming pool E	Water	T4	5+	3+	—	—	5+	4+
4	E_i8	Swimming pool E	Water	T4	6+	3+	—	—	4+	+

5	F_i1	Swimming pool F	Water	T4	6+	3+	–	–	3+	+
6	F_i3	Swimming pool F	Water	T4	6+	5+	–	–	5+	2+
7	B_i1	Swimming pool B	Water	T4	6+	5+	–	–	5+	2+
8	B_i3	Swimming pool B	Water	T4	6+	4+	–	–	5+	5+
9	C_i1	Swimming pool C	Water	T4	6+	5+	–	–	4+	2+
10	C_i3	Swimming pool C	Water	T4	7+	4+	–	–	4+	+
11	D_i1	Swimming pool D	Water	T4	6+	5+	–	–	4+	+
12	D_i2	Swimming pool D	Water	T4	6+	2+	–	–	3+	+

13	HC1_Soil_s2	Onion farmland in HengChun	Soil	T4	7+	7+	—	—	5+	—
14	HC1_Soil_s5	Onion farmland in HengChun	Soil	T4	6+	7+	5+	5+	3+	—
15	HC2_Soil_s1	Onion farmland in HengChun	Soil	T4	5+	4+	—	—	5+	—
16	HC2_Soil_s3	Onion farmland in HengChun	Soil	T4	6+	7+	—	—	5+	—
17	HC2_Soil_s4	Onion farmland in HengChun	Soil	T4	4+	3+	3+	2+	3+	—
18	HC2_Soil_s5	Onion farmland in HengChun	Soil	T5	6+	6+	5+	3+	3+	—
19	HC2_Soil_s6	Onion farmland in HengChun	Soil	T4	5+	3+	—	—	4+	+
20	CC1_Soil_s1	Onion farmland in CheCheng	Soil	T5	7+	5+	5+	4+	4+	—
21	CC1_Soil_s5	Onion	Soil	T5	6+	6+	3+	3+	2+	—

		farmland in CheCheng								
22	CC2_Soil_s1	Onion farmland in CheCheng	Soil	T4	6+	6+	5+	–	3+	–
23	CC2_Soil_s2	Onion farmland in CheCheng	Soil	T4	6+	6+	5+	–	3+	–
24	CC2_Soil_s3	Onion farmland in CheCheng	Soil	T5	6+	6+	5+	3+	3+	–
25	CC2_Soil_s4	Onion farmland in CheCheng	Soil	T4	5+	6+	5+	4+	3+	–
26	HC1_Air_s4	Onion farmland in HengChun	Air	T4	6+	–	–	–	3+	+
27	HC2_Air_s3	Onion farmland in HengChun	Air	T15	4+	5+	–	–	–	–
28	HC2_Air_s6	Onion farmland in HengChun	Air	T4	3+	6+	–	–	3+	–
REFERENCE STRAINS										

1	*Acanthamoeba polyphaga* (ATCC 30461) from *Acanthamoeba* keratitis[b]	4+	4+	─	─	4+	4+
2	*Acanthamoeba castellanii* (ATCC 30234) from Yeast culture[c]	3+	2+	─	─	2+	+

a Score: with number of *Acanthamoeba*/microscopic field counts of zero being scored as ─, one to14 being scored as +, 15 to 29 being scored as 2+, 30 to 59 being scored as 3+, 60 to 89 being scored as 4+, 90 to 149 being scored as 5+, 150 to 299 being scored as 6+, 300 to 500 being scored as 7+, respectively (n=3)

b *Acanthamoeba polyphaga* (ATCC 30461), isolated from *Acanthamoeba* keratitis, genotype T4 (Thompson et al., 2008), was used as a reference of potentially pathogenic isolate

c *Acanthamoeba castellanii* (ATCC 30234), isolated from yeast culture, was used as a reference examples of probably pathogenic isolates (Maghsood et al., 2005)

5.3. Environmental factors

5.3.1. Swimming pool

To evaluate the factors associated with *Acanthamoeba* potentially pathogenicity, the environmental factors of six swimming pools were studied. Table 21 indicates the average values and ranges of environmental factors from water sampled from six swimming pools.

The highest value of free residual chlorine was A swimming pool (0.99 mg/L) and the lowest was E swimming pool (0.26 mg/L) among six swimming pools. There were similar in pH and water temperature among six swimming pools. The six swimming pools are all indoor heated swimming pools, therefore the temperature were well-controlled, ranged from 27.43 to 30.48^{o}C. The highest value of turbidity was A swimming pool (0.8 NTU) and lowest was C swimming pool (0.3 NTU) among six swimming pools. The highest value of hardness was E swimming pool (246.67 mg/L as $CaCO_3$) and the lowest was D swimming pool (36.5 mg/L as $CaCO_3$) among six swimming pools. There were similar in dissolved oxygen among six swimming pools, with the highest was in F swimming pool (9.28 mg/L) and the lowest was A swimming pool (7.21 mg/L). The highest value of salinity was F swimming pool (0.5‰) and the five swimming pools were 0.2 to 0.3‰. The highest value of conductivity was F swimming pool (987.83 μS/cm) and the lowest was JhongJheng sports center swimming pool (437.5 μS/cm) among six swimming pools. The highest value of total dissolved solids was F swimming pool (484.5 mg/L as NaCl) and the lowest was D swimming pool

(211.83 mg/L as NaCl) among six swimming pools. As for heterotrophic plate count, the highest concentration was E swimming pool (4591.67 CFU/mL) and the lowest was D swimming pool (125.42 CFU/mL) among six swimming pools. The population density in A swimming pool was the highest among six swimming pools (0.112 people/ m^2) and population density in D swimming pool was the lowest (0.048 people/ m^2).

Table 21 Environmental factors of water samples from six swimming pools

Environmental factors	Arithmetic mean ± SD (Range)					
	A-SP[a]	E-SP[b]	F-SP[c]	B-SP[d]	C-SP[e]	D-SP[f]
Free residual chlorine (mg/L) (n=6)	0.99±0.10 (0.85-1.11)	0.26±0.10 (0.18-0.38)	0.35±0.01 (0.34-0.36)	0.50±0.06 (0.45-0.59)	0.61±0.13 (0.42-0.76)	0.65±0.16 (0.58-0.87)
pH (n=6)	7.22±0.07 (7.09-7.28)	7.40±0.24 (6.92-7.55)	7.30±0.03 (7.25-7.34)	7.17±0.02 (7.15-7.19)	7.27±0.02 (7.24-7.3)	7.01±0.02 (6.98-7.04)
Water temperature (^{o}C) (n=6)	29.98±0.15 (29.8-30.2)	30.48±0.04 (30.4-30.5)	27.43±0.22 (27.1-27.7)	28.18±0.48 (27.3-28.7)	28.52±0.24 (28.2-28.9)	27.93±0.46 (27.1-28.3)
Turbidity (NTU) (n=6)	0.80±0 (0.8)	0.48±0.08 (0.4-0.6)	0.69±0.06 (0.58-0.74)	0.42±0.11 (0.28-0.56)	0.30±0.03 (0.25-0.34)	0.38±0.05 (0.32-0.44)
Hardness (mg/L as $CaCO_3$) (n=6)	53.33±7.97 (40-62)	246.67±8.16 (230-250)	42.83±12.45 (29-56)	90.50±5.96 (83-97)	71.83±9.00 (61-86)	36.50±6.80 (26-44)
Dissolved oxygen (mg/L) (n=6)	7.21±0.08 (7.1-7.3)	7.3±0.06 (7.21-7.36)	9.28±0.12 (9.1-9.4)	8.05±0.48 (7.66-8.96)	8.65±0.21 (8.26-8.86)	8.81±0.07 (8.72-8.89)
Salinity (‰)(n=6)	0.20±0 (0.2)	0.30±0 (0.3)	0.50±0 (0.5)	0.30±0 (0.3)	0.30±0.03 (0.3)	0.20±0 (0.2)

Conductivity (μS/cm) (n=6)	570.33±2.58 (567-574)	780.33±1.03 (779-782)	987.83±0.98 (987-989)	705.67±4.50 (701-711)	640/33±2.34 (639-645)	437.5±2.95 (435-442)
Total dissolved solids (mg/L as NaCl) (n=6)	276.33±1.21 (275-278)	381±0.63 (380-382)	484.50±0.55 (484-485)	343.67±2.25 (341-346)	311.50±1.22 (311-314)	211.83±1.17 (211-214)
Heterotrophic plate count (CFU/mL) (n=6)	717.08±83.21 (642-722.5)	4591.67±476.1 (4000-5375)	489.17±21.72 (462.5-525)	235.83±51.78 (152.5-275)	432.50±14.32 (410-445)	125.42±19.46 (95-152.5)
Population density (people/m^2) (n=1)	0.112	0.068	0.094	0.071	0.073	0.048

a A swimming pool

b E swimming pool

c F swimming pool

d B swimming pool

e C swimming pool

f D swimming pool

5.3.2. Soil of onion farmlands

Environmental factors of soil were collected during sampling in 2012. Table 22 indicates the values of three environmental factors for soil of nine onion farmlands. The results indicated water content in Hengchun onion farmland 5 (19.04%) was the highest, and the lowest was Hengchun onion farmland 4 (2.19%) among all the onion farmlands. It was noted that the pH of soil was 7.55 in Hengchun onion farmland 1, which was the highest among all the onion farmlands, and the lowest was Checheng onion farmland 4 (6.57). In terms of heterotrophic plate count, Hengchun onion farmland 5 (6.5×10^8 CFU/g) was the highest, and the lowest was Hengchun onion farmland 4 (4.5×10^7 CFU/g) among all the onion farmlands. Overall, the water contents were less in soil and considered as dry soil, the pH were in around 7, and higher concentrations of heterotrophic plate count were in all soils.

Table 22 Environmental factors of soil samples from onion farmlands in HengChun, CheCheng Township (n = 1)

No.	Sampling site	Sampling date (Month/Day/Year)	*Acanthamoeba* isolated ID	Environmental factors		
				Water content (%)	pH	Heterotrophic plate count (CFU/g)
1	Hengchun Onion Farmland1	03/20/2012	HC2_Soil_s1	9.22	7.55	7×10^7

2	Hengchun Onion Farmland3	03/21/2012	HC2_Soil_s3	5.61	7.38	1.1×10^8
3	Hengchun Onion Farmland4	03/21/2012	HC2_Soil_s4	2.19	7.28	4.5×10^7
4	Hengchun Onion Farmland5	03/22/2012	HC2_Soil_s5	19.04	7.35	6.5×10^8
5	Hengchun Onion Farmland6	03/22/2012	HC2_Soil_s6	15.01	7.47	1×10^8
6	Checheng Onion Farmland1	03/19/2012	CC2_Soil_s1	3.69	7.02	2×10^8
7	Checheng Onion Farmland2	03/23/2012	CC2_Soil_s2	8.14	7.29	8×10^7
8	Checheng Onion Farmland3	03/23/2012	CC2_Soil_s3	3.05	6.64	2.4×10^8
9	Checheng Onion Farmland4	03/24/2012	CC2_Soil_s4	6.68	6.57	1.85×10^8

5.3.3. Ambient air of onion farmlands

Environmental factors of ambient air were collected during sampling in 2012.Table 23 indicates the values of environmental factors in the ambient air of 10 onion farmlands. Temperature in Hengchun onion farmland 2 (31.43^{o}C) was the highest and temperature in Checheng onion farmland 4 (20.13^{o}C) was the lowest among all onion farmlands. Wind velocity in Hengchun onion farmland 4 (6.31 m/s) was the highest and in Hengchun onion farmland 1 (1.48 m/s) was the lowest among all onion farmlands. Relative humidity in Hengchun Onion Farmland 6 (72.08 %) was the highest and in Hengchun Onion Farmland 2 (54.38%) was the lowest among all onion farmlands.

Table 23 Environmental factors of the ambient air of onion farmlands in HengChun, CheCheng Township (n = 6)

No.	Sampling site	Sampling date (Month/Day/Year)	*Acanthamoeba* isolated ID	Temperature (^{o}C)	Wind velocity (m/s)	Relative humidity (RH, %)
				Arithmetic mean ± SD (Range)	Arithmetic mean ± SD (Range)	Arithmetic mean ± SD (Range)
1	Hengchun Onion Farmland1	03/20/2012	ND[a]	29.05 ± 0.65 (28.1 – 29.9)	1.48 ± 0.73 (1.02 – 2.92)	64.93 ± 2.48 (62.6 – 69.3)
2	Hengchun Onion Farmland2	03/20/2012	ND	31.43 ± 2.24 (28.8 – 34.3)	1.58 ± 0.77 (0.48 – 2.68)	54.38 ± 7.71 (45.1 – 63.7)
3	Hengchun Onion Farmland3	03/21/2012	HC2_Air_s3	27.32 ± 0.93 (26.1 – 28.6)	2.53 ± 0.64 (1.29 – 3.06)	56.62 ± 3.18 (52.5 – 60.3)
4	Hengchun Onion Farmland4	03/21/2012	ND	25.38 ± 2.08 (22.5 – 28)	6.31 ± 0.34 (5.77 – 6.69)	64.08 ± 7.15 (55.9 – 74.7)
5	Hengchun Onion Farmland5	03/22/2012	ND	29.03 ± 0.52 (28.3 – 29.9)	5.05 ± 0.66 (3.79 – 5.5)	62.15 ± 1.13 (60.8 – 63.8)
6	Hengchun Onion Farmland6[b]	03/22/2012	HC2_Air_s6	27.14 ± 1.35 (25.5 – 28.9)	3.42 ± 0.96 (2.26 – 4.93)	72.08 ± 5.71 (65.1 – 78.8)

7	Checheng Onion Farmland1	03/19/2012	ND	30.45 ± 1.37 (28.2 – 31.7)	2.68 ± 0.27 (2.33 – 3)	55.97 ± 4.67 (51.4 – 64.1)
8	Checheng Onion Farmland2	03/23/2012	ND	31.02 ± 0.90 (29.6 – 32)	1.81 ± 0.64 (1.39 – 2.92)	63 ± 2.24 (60.9 – 67)
9	Checheng Onion Farmland3	03/23/2012	ND	31.1 ± 1.66 (29.4 – 33.5)	2.07 ± 0.65 (1.28 – 2.8)	66.75 ± 5.85 (58.4 – 73.5)
10	Checheng Onion Farmland4	03/24/2012	ND	20.13 ± 0.73 (19.3 – 21.4)	5.84 ± 0.55 (5 – 6.4)	68.55 ± 2.14 (65.9 – 72.2)

[a] Non-detectable for *Acanthamoeba*

[b] Temperature, wind velocity, and relative humidity in Hengchun Township, farmland 6 at 150 min were not collected

Chapter 6 Discussion

6.1. Quality assurance and quality control on thermotolerance and osmotolerance pretesting

6.1.1. Thermotolerance

A.polyphaga and *A.castellanii* were able to grow at 37^{o}C (Table 10 and Table 11). The maximum number of *A.polyphaga* (69 cells/microscopic field) occurred at 9th day of incubation at the distance ranging from 15.36 to 24.82 mm away from the plate center (Table 10 and Fig. 13). As for *A.castellanii*, it was found on 8thday of incubation at the distance ranging from 26.00 to 35.45 mm away from the plate center, with the number of 54 cells/microscopic field (Table 11 and Fig. 14). Neither *A.polyphaga* nor *A.castellanii* was detected at 42^{o}C incubation. To investigate the effects of higher temperatures on amoebic viability, *A.polyphaga* and *A.castellanii* were exposed to 52 ^{o}C for 24 hours prior to incubations at 30^{o}C, which favored for amoebic excystation. The test revealed that *A.polyphaga* and *A.castellanii* could not tolerate and migrate at 52^{o}C. Most studies on evaluation of thermotolerance isolated *Acanthamoeba* were based on the presence or absence of *Acanthamoeba* at 37^{o}C or higher incubation temperatures; however, the observation area on agar was not mentioned (Gianinazzi et al., 2010; Kilic et al., 2004; Lorenzo-Morales et al., 2006). In the present study, quantitative data was provided since we controlled the initial number of *Acanthamoeba* (1000 cells) on each NNA plate and counted the number of *Acanthamoeba* on the

10^{th} day after incubation at a given area (15.36 to 24.82 mm away from the center plate) of the agar plate, which proved a better indicator on thermotolerance testing, similar to the study designed by Caumo et al. (2009). Caumo et al. (2009), used *A. castellanii* Neff (ATCC 30010) isolated from soil environment (Neff) as the probably nonpathogenic strain and another clinical (T4) strain of *A. castellanii* (ATCC 50492) isolated from AK patient as the potentially pathogenic isolate in their thermotolerance and osmotolerance assay. They reported that the number of Neff strain and T4 strains was greater than 30 cysts and/or trophozoites at 37^{o}C, as for 42^{o}C incubation, Neff strain revealed no growth and T4 strain revealed 16 to 30 cysts and/or trophozoites. Pathogenic T4 strain indicated higher thermotolerance at 42^{o}C than non-pathogenic Neff strain.

In the present study, *A. polyphaga* (ATCC 30461) and *A. castellanii* (ATCC 30234) were represented as potentially pathogenic strain and probably pathogenic strain, respectively. *A. polyphaga* (ATCC 30461) was isolated from AK patient and therefore identified as potentially pathogenic strain in the present study. The cytotoxicity determined using LDH assays revealed 51.5 ± 4.5 reported by Maghsood et al. in 2005, and thus recognized *A. castellanii* (ATCC 30234) as probably pathogenic strain. Our results on thermotolerance showed both *A. polyphaga* (ATCC 30461) and *A. castellanii* (ATCC 30234) stains can grow at 37^{o}C (Table 10 and Table 11), which is similar with Caumo et al. (2009). However, both *A. polyphaga* (ATCC 30461) and *A. castellanii* (ATCC 30234) stains in present study cannot grow at 42^{o}C, which is different from Caumo et al.

(2009), the Neff strain revealed no growth and T4 strain revealed 16 to 30 cysts and/or trophozoites. The growth ability difference between *Acanthamoeba* strains isolated from AK patient at 42^{o}C may attribute to temperature tolerance decrease after long-term axenic culture at room temperature (Pumidonming et al., 2010).

6.1.2. Osmotolerance

A. polyphaga and *A.castellanii* grew well without addition of mannitol. *A. polyphaga* grew better than *A. castellanii* at 1M mannitol, the maximum number of *A. polyphaga* (78 cells/microscopic field) was found at 10th day of incubation at the distance ranging from 15.36 to 24.82 mm away from the plate center (Table 12 and Fig.15). As for *A. castellanii*, the maximum number (5 cells/microscopic field) was found at 10th day of incubation at the distance from 15.36 to 24.82 mm away from the plate center (Table 13 and Fig. 16).

Similar to thermotolerance, most previous studies on evaluation of osmotolerance of isolated *Acanthamoeba* were based on the presence or absence of *Acanthamoeba* at 1 M of mannitol; and the author did not mention the observation area on agar (Gianinazzi et al., 2010; Kilic et al., 2004; Lorenzo-Morales et al., 2006). In the present study, we provide the quantitative data by controlling the initial number of *Acanthamoeba* and counting the number of *Acanthamoeba* at 10th day after incubation at a fixed area (15.36 to 24.82 mm) of the agar plate, similar to the study by Caumo et al.

(2009). *A. castellanii* Neff (ATCC 30010) as probably nonpathogenic and clinical (T4) strains of *A. castellanii* (ATCC 50492) as pathogenic strains showed that 16 to 30 cysts and greater than 30 and/or trophozoites were observed for Neff strain and T4 strain, respectively at 0.5 M mannitol. As for testing at 1 M mannitol, Neff strain revealed no growth and T4 strain revealed 16 to 30 cysts and/or trophozoites. Their results show that pathogenic T4 strain higher osmotolerance at 1 M mannitol than non-pathogenic Neff strain. Our results on osmotolerance showed that although both *A. polyphaga* (ATCC 30461) and *A. castellanii* (ATCC 30234) stains can grow at 0.5 M mannitol, the number of *Acanthamoeba* cells per microscopic field of *A. polyphaga* were greater than *A. castellanii*, indicated *A. polyphaga* grew better than *A. castellanii* at 1 M mannitol (Table12 and Table 13). This is similar with Caumo et al. (2009), T4 strain revealed 16 to 30 cysts and/or trophozoites while Neff strain revealed no growth of cysts and/or trophozoites. Overall, Acanthamoeba strains isolated from AK patients in our study (*A. polyphaga*) and in Caumo et al. (2009) (T4 strain) can grew at 1 M mannitol, which is also correlated with studies revealed pathogenic *Acanthamoeba* can withstand osmolarity similar to human eyes (Khan et al., 2001).

6.2. Environmental investigation

6.2.1. Axenic culture and Xenic culture

Xenic culture by sterilized PAS with heat-killed *E.coli* rather than axenic culture by PYG

medium were used to proliferate *Acanthamoeba* isolates in present study since the difficulty in axenizing *Acanthamoeba* isolates using PYG medium for amoeba-positive samples. Due to the interference caused by fungi and bacteria. Besides, literature indicates that some *Acanthamoeba* strains are difficult to multiply in PYG medium (Nagyová et al., 2010a; Nagyová et al., 2010b), and mentioned that out of 22 samples obtained from various sites in Tehran, 13 (59%) isolates readily adapted to growth in monoxenic medium and identified as *Acanthamoeba* spp. microscopically. Only two isolate were successfully cultured in an axenic PYG medium (Eftekhar et al., 2010).

6.2.2. Positive rate of *Acanthamoeba* isolates

6.2.2.1. Pool water

The present study shows the presence of *Acanthamoeba* in the water of all six heated indoor swimming pools, surface soil of all 11 onion farmlands in soils and ambient air of three onion farmlands (Table 14). Our observations accorded with the findings by Caumo et al., 2009, mentioning that *Acanthamoeba* can withstand chlorination in pool water with free chlorine level 1.0 – 4.0 mg/L.

In the present study, all water samples from swimming pools detected the presence of *Acanthamoeba* isolations (positive rate = 100%) utilizing microscopy and PCR approaches. A summary of studies on the positive rate for *Acanthamoeba* isolated from swimming pools is

presented on Table 14. Rezaeian et al. (2008) detected 2 positive water samples from six swimming pools in Tehran (Table 24). In Poland, *Acanthamoeba* was detected in more than half of the swimming pools investigated by Gornik and Kuzna-Grygiel (2004), considered frequency of *Acanthamoeba* detection in open-air swimming pools, being attributed not only to the resistance to chlorination but to the atmospheric precipitation and organic pollution of the pools by humans (Table 24). In Finland, only one in 12 swimming pools (8.33%) were found *Acanthamoeba*-positive (Vesaluoma et al., 1995), probably because of the efficient disinfection and cleaning of the sampled pools (Table 24). Rivera et al. (1993) discovered *Acanthamoeba*, with four pathogenic strains most frequently found, in five out of 11 swimming pools (45.5%) in Mexico City, and they attributed their findings to lack of maintenance of adequate hygiene condition in pools (Table 24). The difference in *Acanthamoeba* positive rate in swimming pools between our study and Rezaeian et al. (2008) may attributed to the *Acanthamoeba* identification methods, we utilized microscopy and PCR with primers JDP1/JDP2 to identified *Acanthamoeba*, and the two studies only take microscopy method into account. The difference in *Acanthamoeba* positive rate in swimming pools between our study and Vesaluoma et al. (1995) may not only attributed to the *Acanthamoeba* identification methods, we utilized microscopy and PCR with primers JDP1/JDP2 to identified *Acanthamoeba*, and the two studies only take microscopy method into account, but to the highest free chlorine concentration in swimming pools (3.3 mg/L), which is much higher than our study

from six swimming pools (0.26 – 0.99 mg/L).

The presence of *Acanthamoeba* in swimming pools may be explained by the resistance of their cyst stages to chlorination of the water. In addition, insufficient cleaning and disinfection with scrub the surfaces of pool and the purification installations in the swimming pools are also contributed *Acanthamoeba* in swimming pools. Studies on pathogenic FLA present in swimming pools by De Jonckheere (1979) have shown that the amoebae, especially *Acanthamoeba* spp., are probably introduced into the water from the soil (surrounding grounds) and by humans, and are not permanent residents of the chlorinated water. However, *Acanthamoeba* were probably from the same source since the species and genotype in six swimming pools yield the same in the present study. The *Acanthamoeba* may be more diverse in species and genotype if *Acanthamoeba* were introduced into the water from the surrounding grounds by humans,

Table 24 Summary of studies on the positive rate for *Acanthamoeba* isolated from swimming pools

Country	Sampling site	Positive rate for *Acanthamoeba*	Identifications of *Acanthamoeba*	*Acanthamoeba* spp.	Environmental factors		Reference
					Environmental factors	Value	
Brazil	Swimming pools	13/65 (20%)	Microscopy and PCR with primers JDP1/JDP2	NA[a]	NA	NA	Caumo and Rott, 2011
Switzerland	Heated indoor swimming pool	1/1 (100%)	Microscopy and PCR with primers JDP1/JDP2	*Acanthamoeba lenticulata*	Water temperature	29.1ºC	Gianinazzi et al., 2009
Philippines	Swimming pool	1/1 (100%)	Microscopy and PCR with primers JDP1/JDP2	*Acanthamoeba lenticulata*	NA	NA	Rivera and Adao, 2008
Iran	Swimming pools	2/6 (33.3%)	Microscopy	NA	NA	NA	Rezaeian et al., 2008
Poland	Indoor swimming pools	10/10 (100%)	Microscopy and negative flagellation test	NA	Water temperature in indoor pools	27 – 33ºC	Gornik and Kuzna-Grygiel, 2004

					Chlorine concentration in indoor pools	0.3 – 0.5 μg/mL	
					In one of the indoor pools, i.e. No. 10, water was additionally conditioned with ozone	0.1 mg O_3/dm^3	
	Open-air swimming pools	3/3 (100%)			Water temperature in open-air pools	27 – 30ºC	
Finland	Swimming pools	1/12 (8.33%)	Microscopy	NA	Total plate count (cfu/mL)	0–3000	Vesaluoma et al., 1995
					Combined chlorine (mg/L)	0.05 – 1.5	
					Free chlorine (mg/L)	< 0.05 – 3.3	
					Total chlorine (mg/L)	0.25 – 4.8	

	pH	3.9 – 9.1
	Potassium permanganate index (mg/L)	1.4 – 2.3
	Turbidity (FTU)	0.09 – 5.9
	Urine (mg/L)	< 0.1 – 2.5

[a] NA: not available

6.2.2.2. Soil of onion farmlands

Similar to pool water, we detected 13 soil samples (100%) by using microscopy and PCR approaches. A summary of studies on the positive rate for *Acanthamoeba* isolated from swimming pools is presented on Table 25. Tsvetkova et al. (2004) also discovered *Acanthamoeba* in all 11 soil samples including clay and sand (100%) in Bulgarian. Another study also detected *Acanthamoeba* in all five soil samples (100%) from Tehran University campus and Laleh Park in Tehran City in Tehran (Rezaeian et al., 2008). Followings are some studies revealed that *Acanthamoeba* in almost, but not all soil samples they collected. *Acanthamoeba* genus-specific DNA was detected in 69 of 75 soil samples (97.18%) collected from public parks located in the central area of Sapporo City, Japan in Kawaguchi et al. (2009). Kilic et al. (2004) detected *Acanthamoeba* in almost all of the 28 soil from and around Military Medical Hospital and most of soil samples were taken from plant pots in different departments of the hospital. in Ankara, Turkey. However, *Acanthamoeba* were identified in 43 of 114 (37.7%) soil samples, which were further tested for their potentially pathogenic were done in Lorenzo-Morales et al., 2005 at Tenerife, Canary Islands, Spain.

The difference in *Acanthamoeba* positive rate in soil samples between our study and Lorenzo-Morales et al. (2005) may not only attributed to the *Acanthamoeba* identification methods, both of our study and Lorenzo-Morales et al. (2005) utilized microscopy and PCR in identification of *Acanthamoeba*. JDP1/JDP2 are the primers we utilized, which are different from

Lorenzo-Morales et al. (2005), *A. astronyxis*, *A. divionensis* and *A. polyphaga* specific primer.

Table 25 Summary of studies on the positive rate for *Acanthamoeba* isolated from soil

Country	Sampling site	Positive rate for *Acanthamoeba*	Identifications of *Acanthamoeba*	*Acanthamoeba* spp.	Environmental factors	Reference
Japan	Public parks located in the central area of Sapporo City	69/71 (97%)	Microscopy and PCR with primers JDP1/JDP2	NA[a]	NA	Kawaguchi et al., 2009
Iran	From numerous localities including an eye center, university campus (Tehran University) and Laleh Park in Tehran City	Others: 5/5 (100%) Eye center: 2/2(100%)	Microscopy	NA	NA	Rezaeian et al., 2008
Philippines	Collected in various regions of the Philippines	10/10 (100%)	Microscopy and PCR with primers JDP1/JDP2	*Acanthamoeba lenticulata*	NA	Rivera and Adao, 2008
Spain	soil sources in Tenerife, Canary Islands, Spain	43/114 (37.7%)	Microscopy and PCR with *A. astronyxis*, *A. divionensis* and *A. polyphaga* specific primer	*A. polyphaga*	NA	Lorenzo-Morales et al., 2005
Turkey	Most of soil samples were taken from plant pots the hospital	For samples were not contaminants:	Microscopy and PCR with genus-specific primers	NA	NA	Kilic et al., 2004

	16/16 (100%)

[a] NA: not available

6.2.2.3. Air of onion farmlands

We detected *Acanthamoeba* in three out of 14 air samples (21.43%) from onion farmlands by utilizing microscopy and PCR approaches. A summary of studies on the positive rate for *Acanthamoeba* isolated from swimming pools is presented on Table 26. A survey was carried out over a one-year period to isolate amoebae suspended in the air of Mexico City, and the author reported *A. polyphaga* as the highest abundant strains (Rivera et al., 1994). We also revealed *A. polyphaga* in the air of onion farmlands (Table 14).Overall, previous studies on identifications of *Acanthamoeba* were only based on microscopy, which are different from our study. We utilized microscopy with PCR approaches which reduce the pseudo-negative in *Acanthamoeba*.

Table 26 Summary of studies on the positive rate for *Acanthamoeba* isolated from ambient air

Country	Sampling site	Positive rate for *Acanthamoeba*	Identifications of *Acanthamoeba*	*Acanthamoeba* spp.	Environmental factors		Reference
					Environmental factors	Value	
Mexico	City of San Luis Potosi, Mexico	NA[a]	Microscopy	*A. polyphaga, A. culbertsoni, A. lenticulata, A. astronyxis, A. triangularis, A. triangularis, A. species, A. griffini, A. rhysodes, A. comandoni, A. lugdunensis, A. mauritaniensis* and *A. palestinensis*	NA	NA	Rodriguez-Zaragoza and Magana-Becerra, 1997
Mexico	Mexico City and its suburbs	NA	Microscopy	*A. quina, A. polyphaga, A. castellanii, A. griffini, A. mauritaniensis, A. divionensis, A. triangularis, A. palestinensis, A. rhysodes, A. lugdunensis, A. hatchetti, A. paradivionensis, A. culbertsoni,* and *A. spp.*	NA	NA	Rivera et al., 1994
Mexico	University campus and Cuemanco	NA	Microscopy	*A. castellanii, A. culbertsoni, A. polyphaga*, and *A. astronyxis*	Air temperature	12.1^{o}C, 15.6^{o}C and 16.4^{o}C	Rivera et al., 1987

	Relative humidity	54.08%, 57.6% and 76.14%
	Wind velocity.	1 m/s, 4 m/s and still

[a] NA: not available

6.2.3. Species identification of isolated *Acanthamoeba*

As *Acanthamoeba* isolates originated from the environments could not be confidently identified only with morphological characteristics, the isolates in the present study were identified as belonging to the genus *Acanthamoeba* based on morphological criteria proposed by Page (1967) and further confirmed by PCR with *Acanthamoeba* genus specific primers JDP1 and JDP2 (Schroeder et al., 2001). DNA sequencing was performed for PCR products by primer JDP1 and the data of DNA sequencing were compared with BLAST in NCBI in order to identify the species of isolated *Acanthamoeba*. Three species of *Acanthamoeba* were found in the present study, i.e. *A. polyphaga, A. lenticulata* and *A. jacobsi* (Table 15). All isolates from swimming pools belonged to *A. polyphaga*. None of the previous study revealed that *A. polyphaga* isolated from swimming pools. However, *A. lenticulata* had been isolated from swimming pools in Switzerland and Philippines (Gianinazzi et al., 2009; Rivera and Adao, 2008) (Table 24).

When it comes to the isolates from soil in the present study revealed that six isolates (HC1_Soil_s2, HC2_Soil_s1, HC2_Soil_s3, HC2_Soil_s6, CC2_Soil_s1 and CC2_Soil_s2) belonged to *A. polyphaga* and seven isolates (HC1_Soil_s5, HC2_Soil_s4, HC2_Soil_s5, CC1_Soil_s1, CC1_Soil_s5, CC2_Soil_s3 and CC2_Soil_s4) belonged to *A. lenticulata* (Table 15). *A. polyphaga* and *A. lenticulata* have also been isolated from soil of park (Rivera and Adao, 2008) and soil (Lorenzo-Morales et al., 2005). Rivera and Adao (2008) discovered five out of 10 isolates

belonging to *A. lenticulata* (5/10), followed by *Acanthamoeba* species (4/10) and *A. mauritaniensis* (1/10) in Philippines (Rivera and Adao, 2008). On the other hand, Lorenzo-Morales et al. (2005) revealed that eight in 14 soil isolates were *Acanthamoeba* species (8/14), followed by *A. polyphaga* (7/14) in Spain (Table 25).

As for *Acanthamoeba* isolated from air, we revealed two isolates (HC1_Air_s4 and HC2_Air_s6) belonged to *A. polyphaga*, and the other isolate (HC2_Air_s3) identified as *A. jacobsi*. *A. polyphaga* had been isolated from the air in previous studies (Magana-Becerra, 1997; Rivera et al., 1987; Rodriguez-Zaragoza and Rivera et al., 1994), however, *A. jacobsi* has not been isolated from the air. Rogerson and Detwiler (1999) in South Dakota found protozoa were detected after cultivation in 22 of the 32 air samples collected, and 9 genus *Acanthamoeba* out of 25 different morphotypes of protozoa were isolated since identifications were not made to the species level, and genus *Acanthamoeba* is the most common being species. A study analyzed air samples by microscopy in the city of San Luis Potosi, Mexico found that pathogenic *Acanthamoeba* from the atmosphere by isolated 23 strains of *Acanthamoeba*, 61% of them were non-pathogenic, 31% were non-pathogenic with invasive capacity and 8% were pathogenic to mice (Rodriguez-Zaragoza and Magana-Becerra, 1997). They revealed a variety of *Acanthamoeba* spp. with *A. polyphaga* (5/23) as the most one, followed by *Acanthamoeba culbertsoni* (3/23), *A. lenticulata* (2/23), *Acanthamoeba astronyxis* (2/23), *Acanthamoeba triangularis* (2/23), *Acanthamoeba species* (2/23), *Acanthamoeba*

griffini (2/23), *Acanthamoeba rhysodes* (1/23), *Acanthamoeba comandoni* (1/23), *Acanthamoeba lugdunensis* (1/23), *A. mauritaniensis* (1/23), *Acanthamoeba palestinensis* (1/23). In a study of airborne amoebae in Mexico City, 108 Strains of amoebae that belong to 12 genera and 41 species were isolated, specimens genus *Acanthamoeba* were most abundant and represented by 14 species and *Acanthamoeba polyphaga* showed the highest abundance were reportedly found Rivera et al., 1994. Rivera et al. (1994) found *Acanthamoeba quina, A. polyphaga, A. castellanii, A. griffini, A. mauritaniensis, Acanthamoeba divionensis, A. divionensis, A. triangularis, A. palestinensis, A. rhysodes, Acanthamoeba lugdunensis, Acanthamoeba hatchetti, A. hatchetti, Acanthamoeba paradivionensis* and *A. culbertsoni* in air samples in Mexico City and its suburbs. Rivera et al., 1987 sampling the air University campus and Cuemanco in Mexico City discovered that three of 11 isolates belonged to *A. castellanii* (3/11), followed by *A. culbertsoni* (3/11), *A. polyphaga* (3/11), *A. astronyxis* (2/11) (Table 25). The diversity of various *Acanthamoeba* species from air samples in previous study may suggest that the variation species isolated from air compared to the species isolated from swimming pools. However, only three isolates isolated from air samples in the present study may limit our findings to this implication. This is the first study on air *Acanthamoeba* in Taiwan that proves that *Acanthamoeba* may exist viable in the atmosphere and the possibility of breathing potentially pathogenic *Acanthamoeba* could contribute to human diseases.

6.2.4. Phylogenetic analysis and genotyping

All *Acanthamoeba* isolates in the present study belonged to T4, T5 and T15 (Table 16 and Fig. 44) which are considered potentially pathogenic since T4, T5, and T15 are also the genotypes being isolated from AK and/or GAE patients (Table 2). In the present study, 34 out of 42 isolates belong to T4 genotype (34/42, 80.95%) (Table 16 and Fig. 44). This result is consistent with the assumption that the relative abundance of T4 isolates in environments. Among 17 known genotype classes (T1 to T17), T4 is the most common genotype to be found in the environment worldwide (Booton et al., 2002; Booton et al., 2004; Booton et al., 2005; Lorenzo-Morales et al., 2006). According to the literature (Booton et al. 2005), 94% (83/88) of AK and 79.3% (23/29) of nonkeratitis infections are associated with the commonly occurring genotype T4. Based on this finding, it was assumed that pathogenic *Acanthamoeba* strains would mainly have genotype T4 (Schroeder et al. 2001; Walochnik et al. 2000a, 2000b). The greater abundance of T4 isolates in our environment samples probably reflects their better adaptation to limited growth condition relative to isolates from other genotypes.

In the present study, seven isolates belonged to genotype T5 (7/42, 16.67%) (Table 16 and Fig. 44). Genotype T5, as the second most prevalent *Acanthamoeba* genotype isolated from environmental samples (Booton et al., 2005), has recently been detected in the patients with AK (Spanakos et al., 2006) and disseminated acanthamoebiasis (Barete et al., 2007). This genotype is

also commonly associated with the species *A. lenticulata* (Stothard et al., 1998).

We revealed only one isolate recognized as genotype T15 (1/42, 2.38%) (Table 16 and Fig. 44). The result is similar with the findings by Kao et al. (2011) that the most frequently identified *Acanthamoeba* genotype from two watersheds, Puzih River and Kaoping River, in southern Taiwan was T4 (n=19), followed by T5 (n=8) and T15 (n=3) (Table 27).

In terms of genotypes in different sample type, we revealed that all 26 isolates from swimming pools belonged to genotype T4 (26/26, 100%) (Table 16 and Fig. 44). This finding differed from Caumo and Rott (2011), 17 *Acanthamoeba* from swimming pools in Southern Brazil, and found that nine of 13 isolates were genotype T5, three were genotype T4, and one was T3 (Table 27).

For 13 isolates from soil samples we found, six isolates as genotype T4 (6/13, 46.2%), and other seven isolates as genotype T5 (7/13, 53.8%) (Table 16 and Fig. 44). Similar to our finding, Niyyati et al. (2009) obtained seven *Acanthamoeba* isolates from soils in numerous localities including an eye center, university campus (Tehran University) and Laleh Park in Tehran City, and identified these seven soil isolates as genotype T4. Rivera and Adao. (2008) isolated 10 *Acanthamoeba* from soils in various regions of the Philippines, and identified five isolates recognized as genotype T5, four as genotype T4 and one as T3 (Table 27). Kilic et al. (2004) isolated *Acanthamoeba* from soils in plant pots in Ankara, Turkey, and showed that two *Acanthamoeba* isolates were T4, five were T5, eight were T2 and one was T7 (Table 27).

For three isolates from air samples, we revealed two were T4 (2/3, 66.6%) and one belonged to T15 (1/3, 33.3%) (Table 16 and Fig. 44). This is the first study to show that genotyping *Acanthamoeba* isolates from air samples.

Table 27 Summary of *Acanthamoeba* genotypes isolated from environments

Country	Sampling site	Sample type	Genotypes of *Acanthamoeba*	Reference
Taiwan	Surface water of two watersheds, Puzih River and Kaoping River	Water	T4 (63.3%) T5 (26.7%) T15 (10%)	Kao et al., 2011
Brazil	Swimming pool	Water	T5 (69.2%) T4 (23.1%) T3 (7.7%)	Caumo and Rott, 2011
Iran	Various locations in Iran	Soil	T4 (100%)	Niyyati et al., 2009
Philippines	Various regions of the Philippines	Soil	T5 (50%) T4 (40%) T3 (10%)	Rivera and Adao, 2008
Egypt	Freshwater sources in the Governorates of Alexandria and Behera, in the Nile Delta region, Egypt	Water	T4 (46.7%) T2 (26.7%) T3 (20%) T7 (6.7%)	Lorenzo-Morales et al., 2006
USA and Scotland	Fort Lauderdale beach , Hollywood beach, Hobe beach and beach in Irvine, Scotland	Beach sand Soil	T4 (95%) T5 (5%) T5 (66.7%) T11 (33.3%)	Booton et al., 2005

Turkey	From and around Military Medical Hospital, Ankara, Turkey	Soil	T2 (50%) T3 (37.5%) T4 (12.5%) T7 (6.25%)	Kilic et al., 2004

6.2.5. Thermotolerance and osmotolerance

Our results show all 28 isolates could survive and migrate at 30^{o}C (Table 18 and Table 19). One isolate from swimming pool and one isolate from air sample cannot growth under 37^{o}C (Table 18 and Table 19). As it is already known, *Acanthamoeba* capable of infecting humans must be capable of surviving at 37^{o}C and slightly higher body temperatures (Schuster and Visvesvara 2004). In 2002, Schuster used clinical samples such as cerebrospinal fluid, brain tissue, scrapings from skin, and corneal scrapings to isolate potentially pathogenic FLA belonging to the genus *Acanthamoeba*. From these samples, isolation in non-nutrient agar occurred in one to two days at 37^{o}C, confirming that at this temperature clinical isolates of *Acanthamoeba* grows readily. In terms of temperature tolerance, 37^{o}C might be the most relevant since the temperature of the eye is only around 36^{o}C (Purslow and Wolffsohn, 2007). These previous observations have led us to postulate that all present *Acanthamoeba* isolates can survive and grow in human bodies which have an average body temperature of ± 37◦C, and are therefore considered as potential human pathogenic isolates.

For 42^{o}C incubation, no *Acanthamoeba* was detected at the distance between 15.36 to 24.82

mm. 12 swimming pool isolates, three air isolates and four soil isolates could not be detected under 42^{o}C (Table 18 and Table 19). Only seven soil isolates retained their viability after 24 hours exposure 52^{o}C and migrate on NNA agar at 30^{o}C (Table 18 and Table 19). The resilience of *Acanthamoeba* cysts to extreme growth temperature probably contribute to the ubiquity, abundance and persistent occurrence in soil environments. This physiological characteristic may also enable the cyst to serve as the ideal vehicle for transmitting *Acanthamoeba* infection from environment to human, and their persistence in the human host. For *Acanthamoeba* in soil, raising the temperature in soil could increase the appearance of thermotolerant species, which is better suited when they invade humans or animals. In another work, Schuster and Visvesvera (2004) reported that increasing in global warming concerning regarding the dispersion of parasitic diseases. Increases in environmental temperatures could favor the growth of the thermotolerant strains that are better adapted to the infection of humans.

As for the results of osmotolerance, all 28 isolates grow well and migrated on NNA agar without addition of mannitol (Table 18 and Table 19). All 12 pool isolates, all 13 soil isolates and two air isolates grew and migrated at the agar with 0.5 M mannitol, except for one air isolate from HengChun onion farmland 3 (Table 18 and Table 19). As for the agar with 1 M mannitol, 12 pool isolates and only one air isolate could tolerate and migrate distantly, but all 13 soil isolates could not (Table 18 and Table 19). This finding on osmotolerance test suggests that 12 swimming pools

isolates and one air isolate migrate at 1 M mannitol are high osmotolerant *Acanthamoeba*, and thus could be regarded as potential human keratitis isolates (Table 18 and Table 19). Most studies on *Acanthamoeba* pathogenicity and osmotolerance find a good association using 1M mannitol (Caumo et al., 2009; Khan and Tareen, 2003; Kilic et al., 2004; Lorenzo-Morales et al., 2006). Although the osmolarity given by 1M mannitol in NNA plates used in the present study was not determined, a previous study indicated that it was 0.25 osmolar (i.e. 250 mOsmol/L) (Khan and Tareen, 2003). The average value of tear film osmolarity in normal human was 304.4 mOsmol/L (Gilbard and Rossi 1994; Gilbard 1994).

6.2.6. The potentially pathogenicity of *Acanthamoeba* isolated from the environments

Except for one isolate (A_i4), the other 11 pool isolates are considered pathogenicity potential since they were identified pathogenic genotype T4, osmotolerant using 1 M mannitol in NNA and grew at 37^{o}C. Only one soil isolate (HC2_Soil_s6) is considered as potentially pathogenic since it was identified as genotype T4 and grew at 1 M mannitol and 37^{o}C. There are no pathogenic potential in air isolates, which can either withstand temperature at 37^{o}C or 1 M mannitol.

A correlation between temperature tolerance (Khan et al., 2001; Khan et al., 2002) and pathogenicity, osmotolerance (Khan et al., 2001; Khan et al., 2002; Khan et al., 2003) and

pathogenicity has been demonstrated. For example, Khan et al. (2002) found that some pathogenic T3 and T4 isolates were all osmotolerant (1 M mannitol) and grew at 37^{o}C. Chan et al. (2011) revealed *Acanthamoeba* isolates can survive and grow at 37^{o}C are considered as potential human pathogenic isolates and isolates can survive and grow at 1 M mannitol are regarded as potential human keratitis (Table 28). Gianinazzi et al. (2010) revealed *Acanthamoeba* isolates can survive and grow at 37^{o}C are considered as potential human pathogenic isolates (Table 28). Caumo et al. (2009) revealed that *Acanthamoeba* isolates not only grew at 1 M mannitol but also grew at 42^{o}C, was considered indicative of pathogenicity. Isolates either grew at 42^{o}C but not at 1 M mannitol, or grew with1 M mannitol but did not grow at 42^{o}C were considered to have low pathogenic potential (Table 28). Isolates did not grow at 42^{o}C or at 1 M mannitol, were considered probably non-pathogenic. Lorenzo-Morales et al. (2006) revealed *Acanthamoeba* isolates can survive and grow at 37^{o}C and 1 M mannitol are pathogenic capacity (Table 28). Kilic et al. (2004) revealed *Acanthamoeba* isolates can survive and grow at 37^{o}C and 1 M mannitol are pathogen. Isolates either grew at 37^{o}C but not at 1 M mannitol, or grew with1 M mannitol but did not grow at 37^{o}C were considered as weak pathogen (Table 28).

Table 28 Pathogenicity potential of *Acanthamoeba* isolated from environments by thermotolerance and/or osmotolerance

Thermotolerance	Osmotolerance	Pathogenicity potential determination	Reference
37°C, 42°C, 46°C, and 52°C (overnight prior to incubation at ambient temperature (26±2°C)	1M mannitol (at ambient temperature)	1) *Acanthamoeba* isolates can survive and grow at 37°C are considered as potential human pathogenic isolates 2) *Acanthamoeba* isolates can survive and grow at 1 M mannitol are regarded as potential human keratitis	Chan et al., 2011
37°C and 42°C	NA[a]	1) *Acanthamoeba* isolates can survive and grow at 37°C are considered as potential human pathogenic isolates	Gianinazzi et al., 2010
37°C and 42°C	0.5 or 1 M mannitol (at 30°C)	1) Not only grew at 1 M mannitol but also grew at 42°C, was considered indicative of pathogenicity 2) Either grew at 42°C but not at 1 M mannitol, or grew with1 M mannitol but did not grow at 42°C were considered to have low pathogenic potential 3) Did not grow at 42°C or at 1 M mannitol, were considered probably non-pathogenic	Caumo et al., 2009
37°C	1M mannitol	1) *Acanthamoeba* isolates can survive and grow at 37°C and 1 M mannitol are pathogenic capacity	Lorenzo-Morales et al., 2006

37^{o}C	1M mannitol	1) *Acanthamoeba* isolates can survive and grow at 37^{o}C and 1 M mannitol are pathogen 2) Either grew at 37^{o}C but not at 1 M mannitol, or grew with1 M mannitol but did not grow at 37^{o}C were considered as weak pathogen	Kilic et al., 2004

[a] NA: not available

Acanthamoeba isolates from soil and isolates from ambient air in the same sampling day and same onion farmland revealed difference of DNA base pair (Table 17), genotype, thermotolerance and osmotolerance in the present study. *Acanthamoeba* (HC2_Soil_s3) isolated from soil in Hengchun Township, farmland 3 and *Acanthamoeba* (HC2_Air_s3) isolated from soil in Hengchun Township, farmland 3 revealed 97% in identities and the gaps was 3%. The pathogenicity of these two isolates from the same sampling day and sampling sites revealed the differences in genotypes, but same in thermotolerance and osmotolerance at 1 M mannitol. The genotype of HC2_Soil_s3 was T4 and HC2_Air_s3 was genotype T15. Both two isolates could grew at 37^{o}C but could not grew at 42 ^{o}C and 52 ^{o}C. The number of HC2_Soil_s3 was 301 number/microscopic field at 37^{o}C and HC2_Air_s3 was 94 number/microscopic field at 37^{o}C. As for osmotolerance, HC2_Soil_s3 was 124 number/microscopic field at 0.5 M mannitol and HC2_Air_s3 could not grew at 0.5 M mannitol, both isolates could not grew at 1 M mannitol.

When it comes to sampling in Hengchun Township, farmland 6, soil isolate (HC2_Soil_s6) and air isolate (HC2_Air_s6) revealed 85% in identities and the gaps was 15%. The pathogenicity of these two isolates from the same sampling day and sampling sites revealed the same in genotype, and same in thermotolerance and osmotolerance at 1 M mannitol. The genotype of HC2_Soil_s6 and HC2_Air_s6 were the same genotype T4. The thermotolerance of HC2_Soil_s3 was 45 number/microscopic field at 37^{o}C and HC2_Air_s3 was 94 number/microscopic field at 37^{o}C, both

isolates could not grew at 42 ^{o}C and 52 ^{o}C. As for osmotolerance, HC2_Soil_s3 was 88 number/microscopic field at 0.5 M mannitol and 4 number/microscopic field at 1 M mannitol HC2_Air_s3 was 52 number/microscopic field at 0.5 M mannitol but could not grew at 1 M mannitol.

6.2.7. Relationship between the presence of *Acanthamoeba* and environmental factors

6.2.7.1. Pool water

Water temperature, salinity, availability to food or nutrients and the cyst forming ability are the factors affecting prevalence of *Acanthamoeba* in the environment (De Jonckheere 1991). The occurrence of *Acanthamoeba* in indoor swimming pools may be attributed to the cyst form are amoebic resistance to chlorination, since cysts can resist a variety of chemical agents, including chlorine (Dejonckheere and Vandevoorde, 1976), and incidence of *Acanthamoeba* keratitis is generally high in countries where water is not treated adequately (Ibrahim et al., 2007), probably attributable to insufficient cleaning and disinfection in swimming pools.

In the present study, all six indoor swimming pools discovered the presence of *Acanthamoeba*, for which the concentration of free chlorine were between 0.26 to 0.99 mg/L. Our finding revealed that *Acanthamoeba* presence in free chlorine of 0.99 mg/L is similar to results of Rivera et al.

(1993), they reported that only chlorine concentration higher than 1.5 mg/mL effectively destroyed cyst form of amoebae. Positive results of amoeba isolated from swimming pools water of Szczecin demonstrate that water chlorination with concentration 0.2 to 0.5 μg/mL did not destroy free-living amoebae (Gornik andKuzna-Grygiel, 2004). Caumo et al. (2009) demonstrated *Acanthamoeba* in heated and unheated swimming pools in Brazilian state, while the free chlorine concentration in heated swimming and unheated pools ranged from 1.0 to 4.0 g/L, and no significant effect of free chlorine on the presence of *Acanthamoeba* in a pool ($P > 0.05$ for each). Experimental studies have demonstrated that the sensitivity of pathogenic and non-pathogenic free-living amoebae were varied to chlorination. The pathogenic *A. culbertsoni* is more resistant to chlorine than the avirulent *Acanthamoeba* strain isolated from tap water , pathogenic *A. culbertsoni* showed positive growth after 3-hour contact with 40 μg/mL of chlorine (De Jonckheere and Van de Voorde, 1976). Chlorination eliminates other competitive microbes, and creates better conditions for resistant amoebae (Griffin 1972).

In the present study, pH of 6 indoor swimming pools was ranged from 7.01 to 7.4, which indicated slightly variation in pH parameter when compared to a study by Caumo et al. (2009). Caumo et al. (2009) discovered *Acanthamoeba* in heated (pH = 7.2 to 8.2) and unheated (pH = 7.0 to 8.2) swimming pools in Brazilian state, but no significant correlation in both heated swimming pools and unheated pools. Griffin (1972) suggests that heating and chlorination of water eliminates

other, competitive microbes, which creates better conditions for the resistant amoebae.

The temperature of six indoor swimming pools ranged from 27.4 to 30.5^{o}C, which is favorable for culture of *Acanthamoeba* (30^{o}C) (Khan, 2006). This findings is similar with Caumo et al (2009), who discovered *Acanthamoeba* in heated swimming pools in Brazilian state with temperature from 23 to 35^{o}C, and discovered *Acanthamoeba* in in unheated swimming pools with temperature ranged from 14 to 26^{o}C, and no significant effect of temperatures on the presence of *Acanthamoeba* in a pool ($P > 0.05$ for each). Permanent heating of water in indoor swimming pools is favorable for persisting thermophile forms of amoebae, which can include strains pathogenic to humans; however, Mazur et al. (1995) demonstrated that if kept at 4ºC for as long as 25 years, the cysts do not lose their viability and infectivity.

The range of water hardness of six indoor swimming pools was extreme largely, from 36.5 to 246.7 mg/L as calcium carbonate ($CaCO_3$). An epidemiology-based study conducted in England and Wales by Radford et al. (2002) discovered that hard water was significantly associated with a threefold increase in risk of *Acanthamoeba* keratitis when compared with soft water. The hardness divided into three groups, soft, medium and hard waters (0 – 99, 100 – 199, and 200 or more mg/L as calcium carbonate respectively). All the pool water samples except for one sample from E swimming pool in present study are identified as soft water (0 – 99 mg/L as calcium carbonate) according to the classification by Radford et al (2002). Although only one pool water in E

swimming pools was identified as hard water (200 or more mg/L as calcium carbonate) according to the classification by Radford et al (2002), there were potentially pathogenic *Acanthamoeba* isolated from all six swimming pools.

The conductivity of six indoor swimming pools ranged from 437.5 to 987.8 μS/cm in the present study. Behets et al. (2007) indicated *Acanthamoeba* spp. are able to dominate at conductivity values >2000 μS/cm^2.

Algae, bacteria and fungi are accumulated and proliferate and serve as food source for *Acanthamoeba* and even other free-living organisms. In the present study, water sampled from swimming pools were surprisingly high, may be explained by high concentrations of heterotrophic plate count in these environments enabling the multiplication of *Acanthamoeba,* which correlated with studies. *Acanthamoeba* were found at 93% in water of wastewater treatment plants, along with high concentrations of bacteria. This may indicate the presence of bacteria in a water source is more important for *Acanthamoeba* multiplication than its oxygen content (Tsvetkova et al., 2004). Huang and Hsu. (2010) revealed the significant differences ($p = 0.049935$) between the presence of *Acanthamoeba* and heterotrophic plate count only when combined PCR and culture methods in spring, hot spring, and waste water in northern Taiwan. However, Kao et al. (2011) found no significant ($p = 0.071$) between the presence of *Acanthamoeba* and heterotrophic plate count samples in spring, hot spring, and waste water in northern Taiwan. *Acanthamoeba*-positivity

samples with HPC mean $3.1\times10^4 \pm 3.98\times10^4$ (CFU/mL) in Kao et al. (2011), which were higher than our study, the HPC in our study were ranged from 125.42 ± 19.46 to 4591.67 ± 476.1.

All the water samples were collected from six swimming pools during normal operation. The four community-type swimming pools were all sampling in the morning around 09:00 a.m. to 10:00 a.m. and were three to four hours after opening. The population density in four community-type swimming pools were around 0.048 to 0.112 (people/m^2). Sampling time of two school-type swimming pools were in the afternoon around 02:00 p.m. to 03:00 p.m. The population density in two school-type swimming pools were around 0.068 to 0.094 (people/m^2). All the six swimming pools were sampling in summer and spring. One of the four community-type swimming pools were sampling at July 2 of 2011, the rest community-type swimming pools were sampling at March 21 and March 28 of 2012. The other two school-type swimming pools were sampling at July 16 of 2011 and March 21 of 2012.

The Department of Health, Taipei City Government regulated water quality in swimming pools (Table 29). Comparisons between environmental factors from six swimming pools collected in the present study with the Taipei City Government regulations revealed that the pH in all six swimming pools were all accorded with the regulations. Free chlorine concentrations in four swimming pools were accorded with the regulations. Free chlorine concentration in A swimming pool (0.99 mg/L) was higher than the Taipei City government regulations. Free chlorine concentration in E swimming

pool (0.26 mg/L) was lower than the Taipei City government regulations. Despite the free chlorine concentration was higher or lower than regulations, there were presence of potentially pathogenic *Acanthamoeba* in all six swimming pools in the present study. There were five out of six swimming pools used automatic filtration followed by automatic chlorination for disinfections. Only one school-type, swimming pool E was used automatic filtration followed by human adding chlorine for disinfections, which may contributed to lower free chlorine concentrations than regulations. Although the chlorination automatic filtration were used to disinfections, there were potentially pathogenic *Acanthamoeba* isolated from all six swimming pools, which may probably attributable to insufficient cleaning and disinfection in swimming pools, the pool water seldom (once in many years) or never drain off and refill the clean water.

Table 29 Swimming pool water quality regulations by the Department of Health, Taipei City Government

Environmental factor	Value
pH	6.5 – 8
Free chlorine concentration (ppm)	0.3 – 0.7
Total bacteria (CFU/mL)	< 500 at 37^{o}C for 24 hrs
Coliform	Negative in five samples (each 10 mL) from 100 mL

6.2.7.2. Soil and air of onion farmlands

In the present study, all nine soil samples discovered *Acanthamoeba* among environmental

collecting samples, which may attributed to *Acanthamoeba* is one of the most dominant species in the soil, the sampling depth, and high concentrations of HPC in soils. Rodriguez-Zaragoza et al. (2005) revealed soil sampled taken from the M. Evenari Runoff Research Farm, Avdat, in the Negev Desert, Israel. The result revealed the type 1 amoebae (e.g., *Acanthamoeba* and *Filamoeba* spp.) were the most abundant throughout the study period, and their numbers were significantly higher than those of the other amoeba types (Rodriguez-Zaragoza et al., 2005). The sampling depth of soil were one cm in the present study, which is similar with study indicated that protozoa decreased more rapidly with increasing depth than the other two groups of organisms (bacteria and fungi) examined (Ekelund et al., 2001) and they indicated that the general tendency of bacteria was a decrease in biomass with increasing depth for all groups examined (Ekelund et al., 2001).

There was one soil isolate (HC2_Soil_s6) considered as potentially pathogenic *Acanthamoeba* isolated from farmland 6 in Hengchun Township. The water content in farmland 6 in Hengchun Township is 15.01%, which is the second highest among all soils. A study indicated the result obtained from the field study demonstrated that the total number of protozoa was significantly higher during the wet seasons (winter and spring) than during the dry seasons (Rodriguez-Zaragoza et al., 2005). Another study revealed the ability of *Acanthamoeba* to form cysts, which are tolerant to desiccation probably explains the findings of viable *Acanthamoeba* in low water content samples (Sriram et al., 2008).

There were two out of 10 air samples discovered weak pathogenic potential *Acanthamoeba* among environmental collecting samples. In two *Acanthamoeba* positive air samples, temperatures were 27^{o}C, wind velocity ranged from 2.5 to 3.4 m/s, and relative humidity ranged from 56.6 to 72.1. The occurrence of *Acanthamoeba* in soil and air of onion farmlands may be attributed to the cysts form of *Acanthamoeba,* which was important for the presence, survival in adverse condition, abundance and diversity in atmosphere. Rivera et al. (1994) mentioned the main source of air-borne amoeba was from the soil, and the factors favored the incidence and diversity of the amoeba isolates were wind speed and wind direction, low relative humidity, generation of frequent dust-storms, re-suspension of amoebae by vehicular traffic, and large extensions of bare soil was a factor associated with reduction incidence and diversity of the aerial amoeba. Rodriguez-Zaragoza and Magana-Becerra (1997) indicated the isolations of *Acanthamoeba* were more abundant during the dry season and the sanitary conditions around stands may enhance the proportion of pathogenic strains in the surroundings. The higher incidence of *Acanthamoeba* was found in the suburban and urban zones because they share common sanitary conditions such as lack of pavement, waste disposed directly on the streets, trapped rain water on the streets, and animal feces at less than 100 m around the stations (Rodriguez-Zaragoza et al., 1993).

Acanthamoeba isolates from soil and isolates from ambient air in the same sampling day and same onion farmland revealed difference *Acanthamoeba* (Table 17) since the variations on

genotypes, thermotolerance and osmotolerance in the present study. These variations may attribute to water content, wind velocity and position of biosampler. The lower water content in soil and wind may bring the surface soil, dust, microorganisms including *Acanthamoeba* into ambient air. A study mentioned that formation of bioaerosol not only depends on temperature and humidity and is highly proportional to the concentration of dust in the air (Tsapko et al., 2011).

Air current is important in the dispersal of microorganisms as it carries them over a long distance. In still air the particles with microorganisms tend to settle down, but a gentle air can keep them in suspension for long periods. The biosampler was placed in the center of each sampling farmland in the present study and the *Acanthamoeba* isolates in air from biosampler may not represent isolates from soil in the same farmland.

The presence of weak potential pathogenic *Acanthamoeba* in onion farmlands indicated potential occupational risk for onion farmers. Ocular trauma is known for the risk factors in farmers for AK (Lin et al., 1999; Ma et al., 1981; Manikandan et al., 2004; Sun et al., 2006). Ocular trauma from pieces of onion skin, onion flakes, dusts or plant leaves drifted into their eyes while onion farmers were harvesting and is more likely to occur in autumn tempest season (October to April) with gusty winds. Hwang et al. (2002) demonstrated onion outer scales may significantly damage the cornea of rats in environments with wind velocity exceeds 10 m/sec; such conditions occur often in autumn tempest season. It is speculated that notably increased levels of dangerous fungi

with a likely occurrence of eye trauma during onion-harvest operations would contribute to the incidence of keratitis among onion harvesters in a monsoon area.

Chapter 7 Conclusions and Suggestions

7.1. Conclusions

1) The present research demonstrating the presence of *Acanthamoeba* in 12 out of 12 water samples (100%) from six swimming pools, 13 out of 13 soil samples (100%) and three out of 14 air samples (21.43%) from onion farmlands in Taiwan.
2) All the *Acanthamoeba* isolates from pool water indicated higher potentially pathogenicity since isolates are pathogenic genotype T4 with thermotolerance ability to grow at 37^{o}C and osmotolerance ability to grow at 1 M mannitol.
3) Only one *Acanthamoeba* isolates from soil indicated higher potentially pathogenicity since isolates are pathogenic genotype T4 with thermotolerance ability to grow at 37^{o}C and osmotolerance ability to grow at 1 M mannitol. On the other hand, rest 12 isolates from soils indicated weak pathogenicity since isolates are pathogenic genotype T4 and T5 with thermotolerance ability to grow at 37^{o}C but without osmotolerance ability to grow at 1 M mannitol. In addition, 69% and 58% of soil isolates with higher thermotoleance ability can withstand and grow at 42^{o}C and 52^{o}C for 24 hours, respectively.
4) *Acanthamoeba* isolates from ambient air indicated weak potentially pathogenicity since isolates are pathogenic genotype T4 and T 15 with either thermotolerance ability to grow at 37^{o}C or

osmotolerance ability to grow at 1 M mannitol.

5) In summary, we have investigated man-made environmental and naturally occurring *Acanthamoeba* in Taiwan with the most commonly observed genotype T4 in the environments and *Acanthamoeba* infections. Thus, the results suggest that swimming pools could be a potential source of *Acanthamoeba* infections. Prevention and control strategies for AK include avoiding showering and swimming while wearing contact lenses. Detection of potentially pathogenicity *Acanthamoeba* in all pool water samples emphasizes the need for enhancing the cleaning and disinfections of swimming pools, in order to decrease the health risk and of human.

6) In conclusion, the results of potentially pathogenicity *Acanthamoeba* presence in soils and ambient air in agricultural settings in Pingtung County indicated that the airborne biological factors play in these settings a primary role as occupational risk factors, exerting harmful effects of the health of exposed workers. Even though all the results are not fully comparable because of the pathogenicity characterization differences in methodology, there is no doubt about the final conclusion on the work-related hazard presented by bioaerosol.

7) The presence in the work environment of potentially pathogenicity *Acanthamoeba* indicated occupational risk of work-related diseases in the exposed agricultural workers particularly since the abrasive nature of pieces of onion skin, onion flakes, dusts or plant leaves and sand particles

can provide corneal trauma and are the risk factor highly associated with AK. Thus, technological in monitoring of pathogenicity *Acanthamoeba* in agricultural settings are necessary steps aiming to improve the working conditions and with appropriate education of both workers and ophthalmologists to protect the workers from perilous health hazards. Onion harvesters should utilize eye protective measures such as wear protective spectacles while working to prevent ocular trauma. Once the ocular trauma, seeking medication from local pharmacies for proper diagnosis and treatment is the first priority in order to prevent delay diagnosis might result in blindness. Also, local ophthalmologists should be informed about this endemic ocular disorder and prepare for suitable treatment.

7.2. Suggestions

1) Combinations of quantifications on concentrations and pathogenicity of *Acanthamoeba* in sampling sites would able well characterized the risk to *Acanthamoeba* diseases.
2) The highest osmolarity tested in the present study was 1 M mannitol equals to 0.25 osmolar, equal to 250 mOsmol/L (Khan and Tareen, 2003), which was lower than osmolarity of the tear film (304.4 ± 0.4 mOsmol/L) (Gilbard and Rossi 1994; Gilbard 1994). Therefore, tested conducted at 1.5 M mannitol as osmotolerance ability as a potentially pathogenic indicator.

3) *Acanthamoeba* isolates could test the thermotolerance ability at 37^{o}C and osmotolerance ability at 1.5 M mannitol at the same time in order to simulated the isolates could survive once enter into human eyes.

4) Collections of AK cases in Hengchun and Checheng Township from local hospitals in order to realized if there was presence of pathogenic *Acanthamoeba* besides the weak potentially pathogenic *Acanthamoeba* isolated from ambient air in the present study.

REFERENCE

1. Abe, N., & Kimata, I. (2010). Genotyping of Acanthamoeba Isolates from Corneal Scrapings and Contact Lens Cases of Acanthamoeba Keratitis Patients in Osaka, Japan. *Japanese Journal of Infectious Diseases, 63*(4), 299-301.
2. Alvord, L., Court, J., Davis, T., Morgan, C. F., Schindhelm, K., Vogt, J., & Winterton, L. (1998). Oxygen permeability of a new type of high Dk soft contact lens material. *Optometry and Vision Science, 75*(1), 30-36.
3. (APHA, 2005) Heterotrophic Plate Count. American Public Health Association, Washington, D.C., 2005, 21st Edition, Section 9215.
4. Barete, S., Combes, A., de Jonckheere, J. F., Datry, A., Varnous, S., Martinez, V., . . . Chosidow, O. (2007). Fatal disseminated Acanthamoeba lenticulata infection in a heart transplant patient. *Emerging Infectious Diseases, 13*(5), 736-738.
5. Behets, J., Declerck, P., Delaedt, Y., Verelst, L., & Ollevier, F. (2007). Survey for the presence of specific free-living amoebae in cooling waters from Belgian power plants. *Parasitology Research, 100*(6), 1249-1256.
6. Bharathi, M. J., Ramakrishnan, R., Meenakshi, R., Padmavathy, S., Shivakumar, C., & Srinivasan, M. (2007). Microbial keratitis in South India: Influence of risk factors, climate, and

geographical variation. *Ophthalmic Epidemiology, 14*(2), 61-69.

7. Bonilla-Lemus, P., Ramirez-Bautista, G. A., Zamora-Munoz, C., Ibarra-Montes, M. D., Ramirez-Flores, E., & Hernandez-Martinez, M. D. (2010). Acanthamoeba spp. in domestic tap water in houses of contact lens wearers in the metropolitan area of Mexico City. *Experimental Parasitology, 126*(1), 54-58.
8. Booton, G. C., Joslin, C. E., Shoff, M., Tu, E. Y., Kelly, D. J., & Fuerst, P. A. (2009). Genotypic Identification of Acanthamoeba sp Isolates Associated With an Outbreak of Acanthamoeba Keratitis. *Cornea, 28*(6), 673-676.
9. Booton, G. C., Kelly, D. J., Chu, Y. W., Seal, D. V., Houang, E., Lam, D. S. C., Byers, T. J.
10. Fuerst, P. A. (2002). 18S ribosomal DNA typing and tracking of Acanthamoeba species isolates from corneal scrape specimens, contact lenses, lens cases, and home water supplies of Acanthamoeba keratitis patients in Hong Kong. *Journal of Clinical Microbiology, 40*(5), 1621-1625
11. Booton, G. C., Rogerson, A., Bonilla, T. D., Seal, D. V., Kelly, D. J., Beattie, T. K., . . . Byers, T. J. (2004). Molecular and physiological evaluation of subtropical environmental isolates of Acanthamoeba spp., causal agent of Acanthamoeba keratitis. *Journal of Eukaryotic Microbiology, 51*(2), 192-200.
12. Booton, G. C., Visvesvara, G. S., Byers, T. J., Kelly, D. J., & Fuerst, P. A. (2005). Identification

and distribution of Acanthamoeba species genotypes associated with nonkeratitis infections. *Journal of Clinical Microbiology, 43*(4), 1689-1693.

13. Bowers, B. (1977). Comparison of Pinocytosis and Phagocytosis in Acanthamoeba-Castellanii. *Experimental Cell Research, 110*(2), 409-417.
14. Bowers, B., & Korn, E. D. (1969). Fine Structure of Acanthamoeba Castellanii (Neff Strain) .2. Encystment. *Journal of Cell Biology, 41*(3), 786-&.
15. Bowers, B., & Olszewski, T. E. (1983). Acanthamoeba Discriminates Internally between Digestible and Indigestible Particles. *Journal of Cell Biology, 97*(2), 317-322.
16. Brown, T. J., & Cursons, R. T. M. (1977). Pathogenic Free-Living Amebae (Pfla) from Frozen Swimming Areas in Oslo, Norway. *Scandinavian Journal of Infectious Diseases, 9*(3), 237-240.
17. Butler, T. K. H., Males, J. J., Robinson, L. P., Wechsler, A. W., Sutton, G. L., Cheng, J, Taylor, P, McClellan, K. (2005). Six-year review of Acanthamoeba keratitis in New South Wales, Australia: 1997-2002. *Clinical and Experimental Ophthalmology, 33*(1), 41-46.
18. Byers, T. J., Akins, R. A., Maynard, B. J., Lefken, R. A., & Martin, S. M. (1980). Rapid Growth of Acanthamoeba in Defined Media - Induction of Encystment by Glucose-Acetate Starvation. *Journal of Protozoology, 27*(2), 216-219.
19. Carvalho, F. R. S., Foronda, A. S., Mannis, M. J., Hofling-Lima, A. L., Belfort, R., & de

Freitas, D. (2009). Twenty Years of Acanthamoeba Keratitis. *Cornea, 28*(5), 516-519.

20. Caumo, K., Frasson, A. P., Pens, C. J., Panatieri, L. F., Frazzon, A. P. G., & Rott, M. B. (2009). Potentially pathogenic Acanthamoeba in swimming pools: a survey in the southern Brazilian city of Porto Alegre. *Annals of Tropical Medicine and Parasitology, 103*(6), 477-485.
21. Caumo, K., & Rott, M. B. (2011). Acanthamoeba T3, T4 and T5 in swimming-pool waters from Southern Brazil. *Acta Tropica, 117*(3), 233-235.
22. Chagla, A. H., & Griffith.Aj. (1974). Growth and Encystation of Acanthamoeba-Castellanii. *Journal of General Microbiology, 85*(Nov), 139-145.
23. Chan, L. L., Mak, J. W., Low, Y. T., Koh, T. T., Ithoi, I., & Mohamed, S. M. (2011). Isolation and characterization of Acanthamoeba spp. from air-conditioners in Kuala Lumpur, Malaysia. *Acta Tropica, 117*(1), 23-30.
24. Chang, C. W., Wu, Y. C., & Ming, K. W. (2010). Evaluation of real-time PCR methods for quantification of Acanthamoeba in anthropogenic water and biofilms. *Journal of Applied Microbiology, 109*(3), 799-807.
25. Chen, W. L., Wu, C. Y., Hu, F. R., & Wang, I. J. (2004). Therapeutic penetrating keratoplasty for microbial keratitis in Taiwan from 1987 to 2001. *American Journal of Ophthalmology, 137*(4), 736-743.
26. Cheng, K. H., Leung, S. L., Hoekman, H. W., Beekhuis, W. H., Mulder, P. G. H., Geerards, A. J.

M., & Kijlstra, A. (1999). Incidence of contact-lens-associated microbial keratitis and its related morbidity. *Lancet, 354*(9174), 181-185.

27. Corsaro, D., & Venditti, D. (2010). Phylogenetic evidence for a new genotype of Acanthamoeba (Amoebozoa, Acanthamoebida). *Parasitology Research, 107*(1), 233-238.

28. Dart, J. K. G., Stapleton, F., & Minassian, D. (1991). Contact-Lenses and Other Risk-Factors in Microbial Keratitis. *Lancet, 338*(8768), 650-653.

29. De Jonckheere, J., & van de Voorde, H. (1976). Differences in destruction of cysts of pathogenic and nonpathogenic Naegleria and Acanthamoeba by chlorine. *Appl Environ Microbiol, 31*(2), 294-297.

30. De Jonckheere, J. F. (1991). Ecology of Acanthamoeba. [Review]. *Reviews of Infectious Diseases, 13 Suppl 5*, S385-387.

31. Dejonckheere, J., & Vandevoorde, H. (1976). Differences in Destruction of Cysts of Pathogenic and Nonpathogenic Naegleria and Acanthamoeba by Chlorine. *Applied and Environmental Microbiology, 31*(2), 294-297.

32. Dharmkrong-at Chusattayanond, A., Boonsilp, S., Kasisit, J., Boonmee, A., & Warit, S. (2010). Thai Acanthamoeba isolate (T4) induced apoptotic death in neuroblastoma cells via the Bax-mediated pathway. *Parasitology International, 59*(4), 512-516.

33. Di Cave, D., Monno, R., Bottalico, P., Guerriero, S., D'Amelio, S., D'Orazi, C., & Berrilli, F.

(2009). Acanthamoeba T4 and T15 genotypes associated with keratitis infections in Italy. *European Journal of Clinical Microbiology & Infectious Diseases*, *28*(6), 607-612.

34. Eftekhar, M., Athari, A., Haghighi, A., Mosaffa, N., Shahram, F., & Abadi, A. (2010). Seroprevalence of Acanthamoeba Antibodies in Rheumatoid Arthritis Patients by IFAT, Tehran, Iran 2007. *Iranian Journal of Parasitology, 5*(1), 35-40.

35. Ekelund, F., Ronn, R., & Christensen, S. (2001). Distribution with depth of protozoa, bacteria and fungi in soil profiles from three Danish forest sites. *Soil Biology & Biochemistry, 33*(4-5), 475-481.

36. Ertabaklar, H., Turk, M., Dayanir, V., Ertug, S., & Walochnik, J. (2007). Acanthamoeba keratitis due to Acanthamoeba genotype T4 in a non-contact-lens wearer in Turkey. *Parasitology Research, 100*(2), 241-246.

37. Ettinger, M. R., Webb, S. R., Harris, S. A., McIninch, S. P., Garman, G. C., & Brown, B. L. (2003). Distribution of free-living amoebae in James River, Virginia, USA. *Parasitology Research, 89*(1), 6-15.

38. Fong, C. F., Tseng, C. H., Hu, F. R., Wang, I. J., Chen, W. L., & Hou, Y. C. (2004). Clinical characteristics of microbial keratitis in a University Hospital in Taiwan. *American Journal of Ophthalmology, 137*(2), 329-336.

39. Fuerst, P. A., Booton, G. C., Visvesvara, G. S., & Byers, T. J. (2003). Genotypic identification

of non-keratitis infections caused by the opportunistically pathogenic ameba genus Acanthamoeba. *Journal of Eukaryotic Microbiology, 50*, 512-513.

40. Gast, R. J. (2001). Development of an Acanthamoeba-specific reverse dot-blot and the discovery of a new ribotype. *Journal of Eukaryotic Microbiology, 48*(6), 609-615.
41. Gatti, S., Rama, P., Matuska, S., Berrilli, F., Cavallero, A., Carletti, S., Bruno, A.
42. Maserati, R, Di Cave, D. (2010). Isolation and genotyping of Acanthamoeba strains from corneal infections in Italy. *Journal of Medical Microbiology, 59*(Pt 11), 1324-1330.
43. Gianinazzi, C., Schild, M., Wuthrich, F., Muller, N., Schurch, N., & Gottstein, B. (2009). Potentially human pathogenic Acanthamoeba isolated from a heated indoor swimming pool in Switzerland. *Experimental Parasitology, 121*(2), 180-186.
44. Gianinazzi, C., Schild, M., Zumkehr, B., Wuthrich, F., Nuesch, I., Ryter, R., Schurch, N.
45. Gottstein, B, Muller, N. (2010). Screening of Swiss hot spring resorts for potentially pathogenic free-living amoebae. *Experimental Parasitology, 126*(1), 45-53.
46. Gilbard, J. P. (1994). Human Tear Film Electrolyte Concentrations in Health and Dry-Eye Disease. *International Ophthalmology Clinics, 34*(1), 27-36.
47. Gilbard, J. P., & Rossi, S. R. (1994). A Unique Therapeutic Artificial Tear Formulation. *Lacrimal Gland, Tear Film, and Dry Eye Syndromes, 350*, 465-469.
48. Gornik, K., & Kuzna-Grygiel, W. (2004). Presence of virulent strains of amphizoic amoebae in

swimming pools of the city of Szczecin. *Annals of Agricultural and Environmental Medicine, 11*(2), 233-236.

49. Griffin, J. L. (1972). Temperature tolerance of pathogenic and nonpathogenic free-living amoebas. *Science, 178*(4063), 869-870.

50. Grimm, D., Ludwig, W., Brandt, B. C., Michel, R., Schleifer, K. H., Hacker, J., & Steinert, M. (2001). Development of 18S rRNA-targeted oligonucleotide probes for specific detection of Hartmannella and Naegleria in Legionella - positive environmental samples. *Systematic and Applied Microbiology, 24*(1), 76-82.

51. Hewett, M. K., Robinson, B. S., Monis, P. T., & Saint, C. P. (2003). Identification of a new Acanthamoeba 18S rRNA gene sequence type, corresponding to the species Acanthamoeba jacobsi Sawyer, Nerad and Visvesvara, 1992 (Lobosea : Acanthamoebidae). *Acta Protozoologica, 42*(4), 325-329.

52. Horn, M., Fritsche, T. R., Gautom, R. K., Schleifer, K. H., & Wagner, M. (1999). Novel bacterial endosymbionts of Acanthamoeba spp. related to the Paramecium caudatum symbiont Caedibacter caryophilus. *Environmental Microbiology, 1*(4), 357-367.

53. Houang, E., Lam, D., Fan, D., & Seal, D. (2001). Microbial keratitis in Hong Kong: relationship to climate, environment and contact-lens disinfection. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 95*(4), 361-367.

54. Hsiao, C. H., Lin, H. C., Chen, Y. F., Ma, D. H. K., Yeh, L. K., Tan, H. Y., Huang, S. C. M., Lin, K. K. (2005). Infectious keratitis related to overnight orthokeratology. *Cornea, 24*(7), 783-788.

55. Hsiao, C. H., Yeung, L., Ma, D. H. K., Chen, Y. F., Lin, H. C., Tan, H. Y., Huang, S. C. M., Lin, K. K. (2007). Pediatric microbial keratitis in Taiwanese children - A review of hospital cases. *Archives of Ophthalmology, 125*(5), 603-609.

56. Hsu, B. M., Ma, P. H., Liou, T. S., Chen, J. S., & Shih, F. C. (2009). Identification of 18S ribosomal DNA genotype of Acanthamoeba from hot spring recreation areas in the central range, Taiwan. *Journal of Hydrology, 367*(3-4), 249-254.

57. Huang, S. W., & Hsu, B. M. (2010). Isolation and identification of Acanthamoeba from Taiwan spring recreation areas using culture enrichment combined with PCR. *Acta Tropica, 115*(3), 282-287.

58. Hwang, Y. H., Chou, E. J., Chang, C. W., Chen, C. C., Ho, C. K., Chou, C. L., Lee, Z. Y., Tseng, C. T. (2002). Suspended onion particles and potential corneal injury in onion harvesters. *Archives of Environmental Health, 57*(1), 78-84.

59. Ibrahim, Y. W., Boase, D. L., & Cree, I. A. (2007). Factors affecting the epidemiology of Acanthamoeba keratitis. *Ophthalmic Epidemiology, 14*(2), 53-60.

60. Jager, B. V., & Stamm, W. P. (1972). Brain Abscesses Caused by Free-Living Amoeba

Probably of Genus Hartmannella in a Patient with Hodgkins Disease. *Lancet, 2*(7791), 1343-&.

61. Janitschke, K., Werner, H., & Muller, G. (1980). [Examinations on the occurrence of free-living amoebae with possible pathogenic traits in swimming pools (author's transl)]. *Zentralbl Bakteriol B, 170*(1-2), 108-122.

62. Jones, D. B., Visvesvara, G. S., & Robinson, N. M. (1975). Acanthamoeba-Polyphaga Keratitis and Acanthamoeba Uveitis Associated with Fatal Meningoencephalitis. *Transactions of the Ophthalmological Societies of the United Kingdom, 95*(Jul), 221-232.

63. Kao, P. M., Hsu, B. M., Chen, N. H., Huang, K. H., Huang, C. C., Ji, D. D., Chen, J. S.,

64. Lin, W. C., Huang, S. W., Chiu, Y. C. (2011). Molecular detection and comparison of Acanthamoeba genotypes in different functions of watersheds in Taiwan. *Environmental Monitoring and Assessment*.

65. Kawaguchi, K., Matsuo, J., Osaki, T., Kamiya, S., & Yamaguchi, H. (2009). Prevalence of Helicobacter and Acanthamoeba in natural environment. *Letters in Applied Microbiology, 48*(4), 465-471.

66. Khan, N. A. (2001). Pathogenicity, morphology, and differentiation of Acanthamoeba. *Current Microbiology, 43*(6), 391-395.

67. Khan, N. A. (2003). Pathogenesis of Acanthamoeba infections. *Microbial Pathogenesis, 34*(6),

277-285.

68. Khan, N. A. (2006). Acanthamoeba: biology and increasing importance in human health. *Fems Microbiology Reviews, 30*(4), 564-595.

69. Khan, N. A. (2008). Acanthamoeba and the blood-brain barrier: the breakthrough. *Journal of Medical Microbiology, 57*(9), 1051-1057.

70. Khan, N. A., Jarroll, E. L., & Paget, T. A. (2001). Acanthamoeba can be differentiated by the polymerase chain reaction and simple plating assays. *Current Microbiology, 43*(3), 204-208.

71. Khan, N. A., Jarroll, E. L., & Paget, T. A. (2002). Molecular and physiological differentiation between pathogenic and nonpathogenic Acanthamoeba. *Current Microbiology, 45*(3), 197-202.

72. Khan, N. A., & Tareen, N. K. (2003). Genotypic, phenotypic, biochemical, physiological and pathogenicity-based categorisation of Acanthamoeba strains. *Folia Parasitologica, 50*(2), 97-104.

73. Khunkitti, W., Lloyd, D., Furr, J. R., & Russell, A. D. (1997). Aspects of the mechanisms of action of biguanides on trophozoites and cysts of Acanthamoeba castellanii. *Journal of Applied Microbiology, 82*(1), 107-114.

74. Khunkitti, W., Lloyd, D., Furr, J. R., & Russell, A. D. (1998). Acanthamoeba castellanii: Growth, encystment, excystment and biocide susceptibility. *Journal of Infection, 36*(1), 43-48.

75. Kilic, A., Tanyuksel, M., Sissons, J., Jayasekera, S., & Khan, N. A. (2004). Isolation of

Acanthamoeba isolates belonging to T2, T3, T4 and T7 genotypes from environmental samples in Ankara, Turkey. *Acta Parasitologica, 49*(3), 246-252.

76. Kilvington, S. (1990). Activity of Water Biocide Chemicals and Contact-Lens Disinfectants on Pathogenic Free-Living Amebas. *International Biodeterioration, 26*(2-4), 127-138.

77. Kilvington, S., Gray, T., Dart, J., Morlet, N., Beeching, J. R., Frazer, D. G., & Matheson, M. (2004). Acanthamoeba keratitis: The role of domestic tap water contamination in the United Kingdom. *Investigative Ophthalmology & Visual Science, 45*(1), 165-169.

78. King, C. H., Shotts, E. B., Wooley, R. E., & Porter, K. G. (1988). Survival of Coliforms and Bacterial Pathogens within Protozoa during Chlorination. *Applied and Environmental Microbiology, 54*(12), 3023-3033.

79. Ku, J. Y., Chan, F. M., & Beckingsale, P. (2009). Acanthamoeba keratitis cluster: an increase in Acanthamoeba keratitis in Australia. *Clinical and Experimental Ophthalmology, 37*(2), 181-190.

80. Kunimoto, D. Y., Sharma, S., Garg, P., Gopinathan, U., Miller, D., & Rao, G. N. (2000). Corneal ulceration in the elderly in Hyderabad, south India. *British Journal of Ophthalmology, 84*(1), 54-59.

81. Lackner, P., Beer, R., Broessner, G., Helbok, R., Pfausler, B., Brenneis, C., Auer, H.,

82. Walochnik, J., Schmutzhard, E. (2010). Acute Granulomatous Acanthamoeba Encephalitis in

an Immunocompetent Patient. *Neurocritical Care, 12*(1), 91-94.

83. Lam, D. S. C., Houang, E., Fan, D. S. P., Lyon, D., Seal, D., Wong, E., & Study, H. K. M. K. (2002). Incidence and risk factors for microbial keratitis in Hong Kong: comparison with Europe and North America. *Eye, 16*(5), 608-618.

84. Lanocha, N., Kosik-Bogacka, D., Maciejewska, A., Sawczuk, M., Wilk, A., & Kuzna-Grygiel, W. (2009). The Occurrence Acanthamoeba (Free Living Amoeba) in Environmental and Respiratory Samples in Poland. *Acta Protozoologica, 48*(3), 271-279.

85. Ledee, D. R., Hay, J., Byers, T. J., Seal, D. V., & Kirkness, C. M. (1996). Acanthamoeba griffini - Molecular characterization of a new corneal pathogen. *Investigative Ophthalmology & Visual Science, 37*(4), 544-550.

86. Ledee, D. R., Iovieno, A., Miller, D., Mandal, N., Diaz, M., Fell, J., Fini, M. E., Alfonso, E. C. (2009). Molecular Identification of T4 and T5 Genotypes in Isolates from Acanthamoeba Keratitis Patients. *Journal of Clinical Microbiology, 47*(5), 1458-1462.

87. Lee, J. E., Oum, B. S., Choi, H. Y., Yu, H. S., & Lee, J. S. (2007). Cysticidal effect on Acanthamoeba and toxicity on human keratocytes by polyhexamethylene biguanide and chlorhexidine. *Cornea, 26*(6), 736-741.

88. Leoni, E., Legnani, P., Mucci, M. T., & Pirani, R. (1999). Prevalence of mycobacteria in a swimming pool environment. *Journal of Applied Microbiology, 87*(5), 683-688.

89. Leoni, E., Legnani, P. P., Sabattini, M. A. B., & Righi, F. (2001). Prevalence of Legionella spp. in swimming pool environment. *Water Research, 35*(15), 3749-3753.

90. Liang, S. Y., Ji, D. R., Hsia, K. T., Hung, C. C., Sheng, W. H., Hsu, B. M., Chen, J. S.

91. Wu, M. H., Lai, C. H., Ji, D. D. (2010). Isolation and identification of Acanthamoeba species related to amoebic encephalitis and nonpathogenic free-living amoeba species from the rice field. *Journal of Applied Microbiology, 109*(4), 1422-1429.

92. Lin, S. H., Lin, C. P., Wang, H. Z., Tsai, R. K., & Ho, C. K. (1999). Fungal corneal ulcers of onion harvesters in southern Taiwan. *Occupational and Environmental Medicine, 56*(6), 423-425.

93. Lloyd, D., Turner, N. A., Khunkitti, W., Hann, A. C., Furr, J. R., & Russell, A. D. (2001). Encystation in Acanthamoeba castellanii: Development of biocide resistance. *Journal of Eukaryotic Microbiology, 48*(1), 11-16.

94. Lorenzo-Morales, J., Monteverde-Miranda, C. A., Jimenez, C., Tejedor, M. L., Valladares, B., & Ortega-Rivas, A. (2005). Evaluation of Acanthamoeba isolates from environmental sources in Tenerife, Canary Islands, Spain. *Annals of Agricultural and Environmental Medicine, 12*(2), 233-236.

95. Lorenzo-Morales, J., Morcillo-Laiz, R., Martin-Navarro, C. M., Lopez-Velez, R., Lopez-Arencibia, A., Arnalich-Montiel, F., Maciver, S. K., Valladares, B., Martinez-Carretero,

E. (2011). Acanthamoeba keratitis due to genotype T11 in a rigid gas permeable contact lens wearer in Spain. *Contact Lens & Anterior Eye, 34*(2), 83-86.

96. Lorenzo-Morales, J., Ortega-Rivas, A., Foronda, P., Martinez, E., & Valladares, B. (2005). Isolation and identification of pathogenic Acanthamoeba strains in Tenerife, Canary Islands, Spain from water sources. *Parasitology Research, 95*(4), 273-277.
97. Lorenzo-Morales, J., Ortega-Rivas, A., Martinez, E., Khoubbane, M., Artigas, P., Periago, M. V., . . . Mas-Coma, S. (2006). Acanthamoeba isolates belonging to T1, T2, T3, T4 and T7 genotypes from environmental freshwater samples in the Nile Delta region, Egypt. *Acta Tropica, 100*(1-2), 63-69.
98. Lund, O. E., Stefani, F. H., & Dechant, W. (1978). Amoebic keratitis: a clinicopathological case report. [Case Reports]. *Br J Ophthalmol, 62*(6), 373-375.
99. Lyons, T. B., & Kapur, R. (1977). Limax amoebae in public swimming pools of albany, schenectady, and rensselaer counties, new york: their concentration, correlations, and significance. *Appl Environ Microbiol, 33*(3), 551-555.
100. Ma, P., Willaert, E., Juechter, K. B., & Stevens, A. R. (1981). A Case of Keratitis Due to Acanthamoeba in New-York, New-York, and Features of 10 Cases. *Journal of Infectious Diseases, 143*(5), 662-667.
101. Maghsood, A. H., Sissons, J., Rezaian, M., Nolder, D., Warhurst, D., & Khan, N. A. (2005).

Acanthamoeba genotype T4 from the UK and Iran and isolation of the T2 genotype from clinical isolates. *Journal of Medical Microbiology, 54*(8), 755-759.

102. Manikandan, P., Bhaskar, M., Revathy, R., John, R. K., Narendran, V., & Panneerselvam, K. (2004). Acanthamoeba keratitis - a six year epidemiological review from a tertiary care eye hospital in south India. *Indian J Med Microbiol, 22*(4), 226-230.

103. Marciano-Cabral, F., & Cabral, G. (2003). Acanthamoeba spp. as agents of disease in humans. *Clinical Microbiology Reviews, 16*(2), 273-+.

104. Martinez, A. J. (1982). Acanthamoebiasis and immunosuppression. Case report. [Case Reports]. *J Neuropathol Exp Neurol, 41*(5), 548-557.

105. Martinez, A. J. (1991). Infection of the Central-Nervous-System Due to Acanthamoeba. *Reviews of Infectious Diseases, 13*, S399-S402.

106. Martinez, A. J., & Janitschke, K. (1985). Acanthamoeba, an Opportunistic Microorganism - a Review. *Infection, 13*(6), 251-256.

107. Martinez, A. J., & Visvesvara, G. S. (1997). Free-living, amphizoic and opportunistic amebas. *Brain Pathology, 7*(1), 583-598.

108. Maubon, D., Dubosson, M., Chiquet, C., Yera, H., Brenier-Pinchart, M. P., Cornet, M., Savy, O., Renard, E., Pelloux, H. (2012). A one-step multiplex PCR for Acanthamoeba keratitis diagnosis and quality samples control. *Invest Ophthalmol Vis Sci*.

109. Mazur, T., Hadas, E., & Iwanicka, I. (1995). The Duration of the Cyst Stage and the Viability and Virulence of Acanthamoeba Isolates. *Tropical Medicine and Parasitology, 46*(2), 106-108.

110. Mergeryan, H. (1991). The Prevalence of Acanthamoeba in the Human Environment. *Reviews of Infectious Diseases, 13*, S390-S391.

111. Nagyova, V., Nagy, A., Janecek, S., & Timko, J. (2010). Morphological, physiological, molecular and phylogenetic characterization of new environmental isolates of Acanthamoeba spp. from the region of Bratislava, Slovakia. *Biologia, 65*(1), 81-91.

112. Nagyova, V., Nagy, A., & Timko, J. (2010). Morphological, physiological and molecular biological characterisation of isolates from first cases of Acanthamoeba keratitis in Slovakia. *Parasitology Research, 106*(4), 861-872.

113. Niyyati, M., Lorenzo-Morales, J., Rahimi, F., Motevalli-Haghi, A., Martin-Navarro, C. M., Farnia, S., Valladares, B., Rezaeian, M. (2009). Isolation and genotyping of potentially pathogenic Acanthamoeba strains from dust sources in Iran. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 103*(4), 425-427.

114. Niyyati, M., Lorenzo-Morales, J., Rezaie, S., Rahimi, F., Martin-Navarro, C. M., Mohebali, M., Maghsood, A. H., Farnia, S., Valladares, B., Rezaeian, M. (2010). First report of a mixed infection due to Acanthamoeba genotype T3 and Vahlkampfia in a cosmetic soft contact lens wearer in Iran. [Case Reports Research Support, Non-U.S. Gov't]. *Experimental Parasitology,*

126(1), 89-90.

115. Niyyati, M., Lorenzo-Morales, J., Rezaie, S., Rahimi, F., Mohebali, M., Maghsood, A. H., Motevalli-Haghi, A., Martin-Navarro, C. M., Farnia, S., Valladares, B.. . . Rezaeian, M. (2009). Genotyping of Acanthamoeba isolates from clinical and environmental specimens in Iran. *Experimental Parasitology, 121*(3), 242-245.

116. Nuprasert, W., Putaporntip, C., Pariyakanok, L., & Jongwutiwes, S. (2010). Identification of a Novel T17 Genotype of Acanthamoeba from Environmental Isolates and T10 Genotype Causing Keratitis in Thailand. *Journal of Clinical Microbiology, 48*(12), 4636-4640.

117. Ofori-Kwakye, S. K., Sidebottom, D. G., Herbert, J., Fischer, E. G., & Visvesvara, G. S. (1986). Granulomatous brain tumor caused by Acanthamoeba. Case report. [Case Reports]. *Journal of Neurosurgery, 64*(3), 505-509.

118. Ozkoc, S., Tuncay, S., Delibas, S. B., Akisu, C., Ozbek, Z., Durak, I., & Walochnik, J. (2008). Identification of Acanthamoeba genotype T4 and Paravahlkampfia sp from two clinical samples. *Journal of Medical Microbiology, 57*(3), 392-396.

119. Page, F. C. (1967). Re-definition of the genus Acanthamoeba with descriptions of three species. *Journal of Protozoology, 14*(4), 709-724.

120. Page, F. C. (1988) A New Key to Freshwater and Soil Gymnamoebae with instructions for culture. Freshwater Biological Association, The Ferry House, Ambleside, Cumbria, UK.

121. Parija, S. C., Prakash, M. R., Rao, V. A., & Vellaniparambil, R. J. (2001). Acanthamoeba keratitis in Pondicherry. *J Commun Dis, 33*(2), 126-129.

122. Patel, D. V., Rayner, S., & McGhee, C. N. J. (2010). Resurgence of Acanthamoeba keratitis in Auckland, New Zealand: a 7-year review of presentation and outcomes. *Clinical and Experimental Ophthalmology, 38*(1), 15-20.

123. Prashanth, K., Pasricha, G., & Sharma, S. (2011). Fluorescence amplified fragment length polymorphism for subtyping of genotypes of Acanthamoeba isolated from patients with keratitis. *Indian Journal of Medical Research, 133*(1), 83-87.

124. Pumidonming, W., Koehsler, M., & Walochnik, J. (2010). Acanthamoeba strains show reduced temperature tolerance after long-term axenic culture. *Parasitology Research, 106*(3), 553-559.

125. Purslow, C., & Wolffsohn, J. (2007). The relation between physical properties of the anterior eye and ocular surface temperature. *Optometry and Vision Science, 84*(3), 197-201.

126. Pussard, M. & Pons, R. (1977). "Morphology de la paroi kystique et taxonomie du genre Acanthamoeba (Protozoa, Amoebida). *Protistologica*, 8, 557-598.

127. Rabi, A., Khader, Y., Alkafajei, A., & Aqoulah, A. A. (2007). Sanitary conditions of public swimming pools in Amman, Jordan. *Int J Environ Res Public Health, 4*(4), 301-306.

128. Radford, C. F., Lehmann, O. J., Dart, J. K. G., & Grp, N. A. K. S. (1998). Acanthamoeba keratitis: multicentre survey in England 1992-6. *British Journal of Ophthalmology, 82*(12),

1387-1392.

129. Radford, C. F., Minassian, D. C., & Dart, J. K. G. (2002). Acanthamoeba keratitis in England and Wales: incidence, outcome, and risk actors. *British Journal of Ophthalmology, 86*(5), 536-542.

130. Reasoner, D. J., & Geldreich, E. E. (1985). A New Medium for the Enumeration and Subculture of Bacteria from Potable Water. *Applied and Environmental Microbiology, 49*(1), 1-7.

131. Rezaeian, M., Niyyati, M., Farnia, S. H., & Haghi, A. M. (2008). Isolation of Acanthamoeba spp. from different environmental sources. *Iranian Journal of Parasitology, 3*(1), 44-47.

132. Ringsted, J., Valjager, B., Suk, D., & Visvesvara, G. S. (1976). Probable Acanthamoeba Meningoencephalitis in a Korean Child. *American Journal of Clinical Pathology, 66*(4), 723-730.

133. Rivera, F., Bonilla, P., Ramirez, E., Calderon, A., Gallegos, E., Rodriguez, S., Ortiz, R., Hernandez, D, V, R. (1994). Seasonal Distribution of Air-Borne Pathogenic and Free-Living Amebas in Mexico-City and Its Suburbs. *Water Air and Soil Pollution, 74*(1-2), 65-87.

134. Rivera, F., Lares, F., Ramirez, E., Bonilla, P., Rodriguez, S., Labastida, A., Ortiz, R., Hernandez, D. (1991). Pathogenic Acanthamoeba Isolated during an Atmospheric Survey in Mexico-City. *Reviews of Infectious Diseases, 13*, S388-S389.

135. Rivera, F., Ortega, A., Lopezochoterena, E., & Paz, M. E. (1979). Quantitative Morphological and Ecological Study of Protozoa Polluting Tap Water in Mexico-City. *Transactions of the American Microscopical Society, 98*(3), 465-469.

136. Rivera, F., Ramirez, E., Bonilla, P., Calderon, A., Gallegos, E., Rodriguez, S., Ortiz, R., Zaldivar, B., Ramirez, P., Duran, A. (1993). Pathogenic and free-living amoebae isolated from swimming pools and physiotherapy tubs in Mexico. *Environ Res, 62*(1), 43-52.

137. Rivera, F., Ramirez, P., Vilaclara, G., Robles, E., & Medina, F. (1983). A survey of pathogenic and free-living amoebae inhabiting swimming pool water in Mexico City. *Environ Res, 32*(1), 205-211.

138. Rivera, F., Roy-Ocotla, G., Rosas, I., Ramirez, E., Bonilla, P., & Lares, F. (1987). Amoebae isolated from the atmosphere of Mexico City and environs. [Research Support, Non-U.S. Gov't]. *Environ Res, 42*(1), 149-154.

139. Rivera, W. L., & Adao, D. E. V. (2008). Identification of the 18S-ribosomal-DNA genotypes of Acanthamoeba isolates from the Philippines. *Annals of Tropical Medicine and Parasitology, 102*(8), 671-677.

140. Rodriguez-Zaragoza, S. (1994). Ecology of free-living amoebae. *Critical Reviews in Microbiology, 20*(3), 225-241.

141. Rodriguez-Zaragoza, S., & Magana-Becerra, A. (1997). Prevalence of pathogenic

Acanthamoeba (Protozoa:Amoebidae) in the atmosphere of the city of San Luis Potosi, Mexico. *Toxicol Ind Health, 13*(4), 519-526.

142. Rodriguez-Zaragoza, S., Mayzlish, E., & Steinberger, Y. (2005). Vertical distribution of the free-living amoeba population in soil under desert shrubs in the Negev Desert, Israel. *Applied and Environmental Microbiology, 71*(4), 2053-2060.

143. Rodriguezzaragoza, S. (1994). Ecology of Free-Living Amebas. *Critical Reviews in Microbiology, 20*(3), 225-241.

144. Rodriguezzaragoza, S., Rivera, F., Bonilla, P., Ramirez, E., Gallegos, E., Calderon, A., Ortiz, R., Hernandez, D. (1993). Amoebological Study of the Atmosphere of San-Luis-Potosi, Slp, Mexico. *Journal of Exposure Analysis and Environmental Epidemiology, 3*, 229-241.

145. Rogerson, A., & Detwiler, A. (1999). Abundance of airborne heterotrophic protists in ground level air of South Dakota. *Atmospheric Research, 51*(1), 35-44.

146. Saeed, A., D'Arcy, F., Stack, J., Collum, L. M., Power, W., & Beatty, S. (2009). Risk Factors, Microbiological Findings, and Clinical Outcomes in Cases of Microbial Keratitis Admitted to a Tertiary Referral Center in Ireland. *Cornea, 28*(3), 285-292.

147. Sangruchi, T., Martinez, A. J., & Visvesvara, G. S. (1994). Spontaneous granulomatous amebic encephalitis: report of four cases from Thailand. [Case Reports

148. Research Support, Non-U.S. Gov't]. *Southeast Asian J Trop Med Public Health, 25*(2),

309-313.

149. Schroeder, J. M., Booton, G. C., Hay, J., Niszl, I. A., Seal, D. V., Markus, M. B., Fuerst, P. A., Byers, T. J. (2001). Use of subgenic 18S ribosomal DNA PCR and sequencing for genus and genotype identification of acanthamoebae from humans with keratitis and from sewage sludge. *Journal of Clinical Microbiology, 39*(5), 1903-1911.

150. Schuster, F. L., & Visvesvara, G. S. (2004a). Amebae and ciliated protozoa as causal agents of waterborne zoonotic disease. *Veterinary Parasitology, 126*(1-2), 91-120.

151. Schuster, F. L., & Visvesvara, G. S. (2004b). Free-living amoebae as opportunistic and non-opportunistic pathogens of humans and animals. *International Journal for Parasitology, 34*(9), 1001-1027.

152. Seal, D. V., Kirkness, C. M., Bennett, H. G., & Peterson, M. (1999). Population-based cohort study of microbial keratitis in Scotland: incidence and features. *Cont Lens Anterior Eye, 22*(2), 49-57.

153. Sharifi, N., Botero-Kleiven, S., Ohman, D., Barragan, A., & Winiecka-Krusnell, J. (2010). Genotypic characterization of Acanthamoeba spp. causing ocular infections in Swedish patients: identification of the T15 genotype in a case of protracted keratitis. *Scandinavian Journal of Infectious Diseases, 42*(10), 781-786.

154. Sharma, S., Garg, P., & Rao, G. N. (2000). Patient characteristics, diagnosis, and treatment of

non-contact lens related Acanthamoeba keratitis. *British Journal of Ophthalmology, 84*(10), 1103-1108.

155. Sharma, S., Pasricha, G., Das, D., & Aggarwal, R. K. (2004). Acanthamoeba keratitis in non-contact lens wearers in India - DNA typing-based validation and a simple detection assay. *Archives of Ophthalmology, 122*(10), 1430-1434.

156. Sharma, S., Srinivasan, M., & George, C. (1990). Acanthamoeba Keratitis in Non-Contact-Lens Wearers. *Archives of Ophthalmology, 108*(5), 676-678.

157. Sheng, W. H., Hung, C. C., Huang, H. H., Liang, S. Y., Cheng, Y. J., Ji, D. D., & Chang, S. C. (2009). Case Report: First Case of Granulomatous Amebic Encephalitis Caused by Acanthamoeba castellanii in Taiwan. *American Journal of Tropical Medicine and Hygiene, 81*(2), 277-279.

158. Singhal, T., Bajpai, A., Kalra, V., Kabra, S. K., Samantaray, J. C., Satpathy, G., & Gupta, A. K. (2001). Successful treatment of Acanthamoeba meningitis with combination oral antimicrobials. *Pediatric Infectious Disease Journal, 20*(6), 623-627.

159. Spanakos, G., Tzanetou, K., Miltsakakis, D., Patsoula, E., Malamou-Lada, E., & Vakalis, N. C. (2006). Genotyping of pathogenic Acanthamoebae isolated from clinical samples in Greece - Report of a clinical isolate presenting T5 genotype. *Parasitology International, 55*(2), 147-149.

160. Srinivasan, M., Burman, S., George, C., & Nirmalan, P. K. (2003). Non-contact lens related Acanthamoeba keratitis at a tertiary eye care center in south India: Implications for eye care programs in the region. [Comparative Study Research Support, Non-U.S. Gov't]. *Med Sci Monit, 9*(4), CR125-129.

161. Sriram, R., Shoff, M., Booton, G., Fuerst, P., & Visvesvara, G. S. (2008). Survival of Acanthamoeba Cysts after Desiccation for More than 20 Years. *Journal of Clinical Microbiology, 46*(12), 4045-4048.

162. Stehr-Green, J. K., Bailey, T. M., & Visvesvara, G. S. (1989). The epidemiology of Acanthamoeba keratitis in the United States. *American Journal of Ophthalmology, 107*(4), 331-336.

163. Stehrgreen, J. K., Bailey, T. M., Brandt, F. H., Carr, J. H., Bond, W. W., & Visvesvara, G. S. (1987). Acanthamoeba Keratitis in Soft Contact-Lens Wearers - a Case-Control Study. *Jama-Journal of the American Medical Association, 258*(1), 57-60.

164. Steinberg, J. P., Galindo, R. L., Kraus, E. S., & Ghanem, K. G. (2002). Disseminated acanthamebiasis in a renal transplant recipient with osteomyelitis and cutaneous lesions: Case report and literature review. *Clinical Infectious Diseases, 35*(5), E43-E49.

165. Storey, M. V., Winiecka-Krusnell, J., Ashbolt, N. J., & Stenstrom, T. A. (2004). The efficacy of heat and chlorine treatment against thermotolerant Acanthamoebae and Legionellae.

Scandinavian Journal of Infectious Diseases, 36(9), 656-662.

166. Stothard, D. R., Schroeder-Diedrich, J. M., Awwad, M. H., Gast, R. J., Ledee, D. R., Rodriguez-Zaragoza, S., Dean, C. L., Fuerst, P. A., Byers, T. J. (1998). The evolutionary history of the genus Acanthamoeba and the identification of eight new 18S rRNA gene sequence types. *Journal of Eukaryotic Microbiology, 45*(1), 45-54.

167. Sun, X. G. (2006). Acanthamoeba keratitis - Author reply. *Ophthalmology, 113*(12), 2377-2377.

168. Sun, X. G., Zhang, Y., Li, R., Wang, Z. Q., Luo, S. Y., Gao, M., Deng, S. J., Chen, W., Jin, X. Y. (2006). Acanthamoeba keratitis - Clinical characteristics and management. *Ophthalmology, 113*(3), 412-416.

169. Tien, S. H., & Sheu, M. M. (1999). Treatment of Acanthamoeba keratitis combined with fungal infection with polyhexamethylene biguanide. [Case Reports]. *Kaohsiung J Med Sci, 15*(11), 665-673.

170. Tsapko, V. G., Chudnovets, A. J., Sterenbogen, M. J., Papach, V. V., Dutkiewicz, J., Skorska, C., Krysinska-Traczyk, E., Golec, M. (2011). Exposure to Bioaerosols in the Selected Agricultural Facilities of the Ukraine and Poland - a Review. *Annals of Agricultural and Environmental Medicine, 18*(1), 19-27.

171. Tseng, S. H., Hu, F. R., Lee, G. S., Chang, S. C., Chen, C. L., & Luh, K. T. (1989). Acanthamoeba keratitis: report of a case. [Case Reports]. *Taiwan Yi Xue Hui Za Zhi, 88*(5),

512-516.

172. Tsvetkova, N., Schild, M., Panaiotov, S., Kurdova-Mintcheva, R., Gottstein, B., Walochnik, J., Aspock, H., Lucas, M. S., Muller, N. (2004). The identification of free-living environmental isolates of amoebae from Bulgaria. *Parasitology Research, 92*(5), 405-413.

173. Turner, N. A., Russell, A. D., Furr, J. R., & Lloyd, D. (2000). Emergence of resistance to biocides during differentiation of Acanthamoeba castellanii. *Journal of Antimicrobial Chemotherapy, 46*(1), 27-34.

174. van der Meulen, I. J., van Rooij, D., Nieuwendaal, C. P., Van Cleijnenbreugel, H., Geerards, A. J., & Remeijer, L. (2008). Age-related risk factors, culture outcomes, and prognosis in patients admitted with infectious keratitis to two Dutch tertiary referral centers. *Cornea, 27*(5), 539-544.

175. Verani, J. R., Lorick, S. A., Yoder, J. S., Beach, M. J., Braden, C. R., Roberts, J. M., Conover, C. S., Chen, S., McConnell, K. A., Chang, D. C., Park, B. J., Jones, D. B., Visvesvara, G. S., Roy, S. L., Team, A. K. I. (2009). National Outbreak of Acanthamoeba Keratitis Associated with Use of a Contact Lens Solution, United States. *Emerging Infectious Diseases, 15*(8), 1236-1242.

176. Vesaluoma, M., Kalso, S., Jokipii, L., Warhurst, D., Ponka, A., & Tervo, T. (1995). Microbiological Quality in Finnish Public Swimming Pools and Whirlpools with Special

Reference to Free-Living Amebas - a Risk Factor for Contact-Lens Wearers. *British Journal of Ophthalmology, 79*(2), 178-181.

177. Visvesvara, G. S. (1993). Epidemiology of Infections with Free-Living Amebas and Laboratory Diagnosis of Microsporidiosis. *Mount Sinai Journal of Medicine, 60*(4), 283-288.

178. Visvesvara, G. S., & Balamuth, W. (1975a). Comparative Studies on Related Free-Living and Pathogenic Amebae with Special Reference to Acanthamoeba. *Journal of Protozoology, 22*(2), 245-256.

179. Visvesvara, G. S., & Balamuth, W. (1975b). Comparative studies on related free-living and pathogenic amebae with special reference to Acanthamoeba. *Journal of Protozoology, 22*(2), 245-256.

180. Visvesvara, G. S., Jones, D. B., & Robinson, N. M. (1975). Isolation, Identification, and Biological Characterization of Acanthamoeba-Polyphaga from a Human Eye. *American Journal of Tropical Medicine and Hygiene, 24*(5), 784-790.

181. Walochnik, J., Aichelburg, A., Assadian, O., Steuer, A., Visvesvara, G., Vetter, N., & Aspock, H. (2008). Granulomatous amoebic encephalitis caused by Acanthamoeba amoebae of genotype T2 in a human immunodeficiency virus-negative patient. *Journal of Clinical Microbiology, 46*(1), 338-340.

182. Walochnik, J., Haller-Schober, E., Kolli, H., Picher, O., Obwaller, A., & Aspock, H. (2000).

Discrimination between clinically relevant and nonrelevant Acanthamoeba strains isolated from contact lens- wearing keratitis patients in Austria. [Case Reports]. *Journal of Clinical Microbiology, 38*(11), 3932-3936.

183. Walochnik, J., Obwaller, A., & Aspock, H. (2000). Correlations between morphological, molecular biological, and physiological characteristics in clinical and nonclinical isolates of Acanthamoeba spp. *Applied and Environmental Microbiology, 66*(10), 4408-4413.

184. Wong, T., Ormonde, S., Gamble, G., & McGhee, C. N. J. (2003). Severe infective keratitis leading to hospital admission in New Zealand. *British Journal of Ophthalmology, 87*(9), 1103-1108.

185. Yera, H., Zamfir, O., Bourcier, T., Viscogliosi, E., Noel, C., Dupouy-Camet, J., & Chaumeil, C. (2008). The genotypic characterisation of Acanthamoeba isolates from human ocular samples. *British Journal of Ophthalmology, 92*(8), 1139-1141.

186. Zhang, Y., Sun, X. G., Wang, Z. Q., Li, R., Luo, S. Y., Jin, X. Y., Deng, S. J., Chen, W. (2004). Identification of 18S ribosomal DNA genotype of Acanthamoeba from patients with keratitis in North China. *Investigative Ophthalmology & Visual Science, 45*(6), 1904-1907.

187. Zhao, G., Sun, S. Y., Zhao, J., & Xie, L. X. (2010). Genotyping of Acanthamoeba isolates and clinical characteristics of patients with Acanthamoeba keratitis in China. *Journal of Medical Microbiology, 59*(4), 462-466.

APPENDIX

APPENDIX A Distance of *Acanthamoeba* ATCC strains migrated after two hours inoculum onto NNA/heat-killed *E.coli*

Numbers of microscopic field away from the center of NNA	Distance away from the center (mm)	Number of trophozoite or cyst on each microscopic field											
		A. polyphaga (ATCC 30461) on 0 M mannitol for two hours			*A.castellanii* (ATCC 30234) on 0 M mannitol for two hours			*A. polyphaga* (ATCC 30461) on 1 M mannitol for two hours			*A.castellanii* (ATCC 30234) on 1 M mannitol for two hours		
		n=1	n=2	n=3	n=1	n=2	n=3	n=1	n=2	n=3	n=1	n=2	n=3
1	1.18	22	16	13	21	12	18	8	8	4	11	19	0
2	2.36	14	14	6	9	13	13	11	5	6	20	15	0
3	3.55	11	13	2	8	7	11	6	1	5	0	15	5
4	4.73	6	6	0	5	4	13	11	3	2	1	21	0
5	5.91	2	3	0	3	0	3	5	14	0	0	13	0
6	7.09	0	0	0	0	0	0	7	3	0	0	3	0
7	8.27	0	0	0	1	0	0	0	0	0	0	0	0
8	9.45	0	0	0	2	0	0	0	0	0	0	0	0
9	10.64	0	0	0	0	0	0	0	0	0	0	0	0
10	11.82	0	0	0	0	0	0	0	0	0	0	0	0
11	13.00	0	0	0	0	0	0	0	0	0	0	0	0
12	14.18	0	0	0	0	0	0	0	0	0	0	0	0
13	15.36	0	0	0	0	0	0	0	0	0	0	0	0
14	16.55	0	0	0	0	0	0	0	0	0	0	0	0
15	17.73	0	0	0	0	0	0	0	0	0	0	0	0
16	18.91	0	0	0	0	0	0	0	0	0	0	0	0

17	20.09	0	0	0	0	0	0	0	0	0	0	0	0
18	21.27	0	0	0	0	0	0	0	0	0	0	0	0
19	22.45	0	0	0	0	0	0	0	0	0	0	0	0
20	23.64	0	0	0	0	0	0	0	0	0	0	0	0
21	24.82	0	0	0	0	0	0	0	0	0	0	0	0
22	26.00	0	0	0	0	0	0	0	0	0	0	0	0
23	27.18	0	0	0	0	0	0	0	0	0	0	0	0
24	28.36	0	0	0	0	0	0	0	0	0	0	0	0
25	29.55	0	0	0	0	0	0	0	0	0	0	0	0
26	30.73	0	0	0	0	0	0	0	0	0	0	0	0
27	31.91	0	0	0	0	0	0	0	0	0	0	0	0
28	33.09	0	0	0	0	0	0	0	0	0	0	0	0
29	34.27	0	0	0	0	0	0	0	0	0	0	0	0
30	35.45	0	0	0	0	0	0	0	0	0	0	0	0

APPENDIX B Results of BLAST of DNA sequences for PCR products of 42 isolates

No.	Sample type	*Acanthamoeba* isolate ID	*Acanthamoeba* species	Accession	Description[a]	Max score[b]	Total score[c]	Query coverage[d]	E value[e]	Max ident[f]
1	Water	A_i4	*Acanthamoeba polyphaga*	AY148954.1	Acanthamoeba sp. KA/E5 18S ribosomal RNA gene, complete sequence	747	747	99%	0.000E+00	99%

				AY173014.1	Acanthamoeba sp. KA/MSS6 18S ribosomal RNA gene, complete sequence	747	747	99%	0.000E+00	99%
				AY173013.1	Acanthamoeba sp. KA/MSS2 18S ribosomal RNA gene, complete sequence	747	747	99%	0.000E+00	99%
				AY026243.1	**Acanthamoeba polyphaga strain ATCC30461 small subunit ribosomal RNA gene, complete sequence**	**747**	**747**	**99%**	**0.000E+00**	**99%**
				JN222978.1	Acanthamoeba sp. 283 18S ribosomal RNA gene, partial sequence	744	744	98%	0.000E+00	99%
				DQ087320.1	Acanthamoeba sp. S4 18S ribosomal RNA gene, partial sequence	744	744	98%	0.000E+00	99%
2	Water	A_i5	*Acanthamoeba polyphaga*	AY148954.1	Acanthamoeba sp. KA/E5 18S ribosomal RNA gene, complete sequence	738	738	96%	0.000E+00	99%
				AY173014.1	Acanthamoeba sp. KA/MSS6 18S ribosomal RNA gene, complete sequence	738	738	96%	0.000E+00	99%

				AY173013.1	Acanthamoeba sp. KA/MSS2 18S ribosomal RNA gene, complete sequence	738	738	96%	0.000E+00	99%
				AY026243.1	**Acanthamoeba polyphaga strain ATCC30461 small subunit ribosomal RNA gene, complete sequence**	**738**	**738**	**96%**	**0.000E+00**	**99%**
				JN222978.1	Acanthamoeba sp. 283 18S ribosomal RNA gene, partial sequence	737	737	96%	0.000E+00	99%
				DQ087320.1	Acanthamoeba sp. S4 18S ribosomal RNA gene, partial sequence	737	737	96%	0.000E+00	99%
3	Water	A_i6	*Acanthamoeba polyphaga*	AY148954.1	Acanthamoeba sp. KA/E5 18S ribosomal RNA gene, complete sequence	746	746	97%	0.000E+00	99%
				AY173014.1	Acanthamoeba sp. KA/MSS6 18S ribosomal RNA gene, complete sequence	746	746	97%	0.000E+00	99%
				AY173013.1	Acanthamoeba sp. KA/MSS2 18S ribosomal RNA gene, complete sequence	746	746	97%	0.000E+00	99%

				AY026243.1	**Acanthamoeba polyphaga strain ATCC30461 small subunit ribosomal RNA gene, complete sequence**	**746**	**746**	**97%**	**0.000E+00**	**99%**
				JN222978.1	Acanthamoeba sp. 283 18S ribosomal RNA gene, partial sequence	742	742	97%	0.000E+00	99%
				DQ087320.1	Acanthamoeba sp. S4 18S ribosomal RNA gene, partial sequence	742	742	97%	0.000E+00	99%
				U07407.1	Acanthamoeba polyphaga BCM:0173:16 ATCC 50371 18S rRNA gene	742	742	97%	0.000E+00	99%
4	Water	A_i7	*Acanthamoeba polyphaga*	AY148954.1	Acanthamoeba sp. KA/E5 18S ribosomal RNA gene, complete sequence	742	742	97%	0.000E+00	99%
				AY173014.1	Acanthamoeba sp. KA/MSS6 18S ribosomal RNA gene, complete sequence	742	742	97%	0.000E+00	99%
				AY173013.1	Acanthamoeba sp. KA/MSS2 18S ribosomal RNA gene, complete sequence	742	742	97%	0.000E+00	99%

				AY026243.1	**Acanthamoeba polyphaga strain ATCC30461 small subunit ribosomal RNA gene, complete sequence**	**742**	**742**	**97%**	**0.000E+00**	**99%**
				JN222978.1	Acanthamoeba sp. 283 18S ribosomal RNA gene, partial sequence	738	738	96%	0.000E+00	99%
				DQ087320.1	Acanthamoeba sp. S4 18S ribosomal RNA gene, partial sequence	738	738	96%	0.000E+00	99%
				U07407.1	Acanthamoeba polyphaga BCM:0173:16 ATCC 50371 18S rRNA gene	738	738	97%	0.000E+00	99%
5	Water	A_i8	*Acanthamoeba polyphaga*	**GU596994.1**	**Acanthamoeba polyphaga isolate A8/SB2 18S ribosomal RNA gene, partial sequence**	**742**	**742**	**96%**	**0.000E+00**	**99%**
				AY148954.1	Acanthamoeba sp. KA/E5 18S ribosomal RNA gene, complete sequence	738	738	99%	0.000E+00	98%
				AY173014.1	Acanthamoeba sp. KA/MSS6 18S ribosomal RNA gene, complete sequence	738	738	99%	0.000E+00	98%

				AY173013.1	Acanthamoeba sp. KA/MSS2 18S ribosomal RNA gene, complete sequence	738	738	99%	0.000E+00	98%
				AY026243.1	Acanthamoeba polyphaga strain ATCC30461 small subunit ribosomal RNA gene, complete sequence	738	738	99%	0.000E+00	98%
6	Water	E_i1	*Acanthamoeba polyphaga*	AY148954.1	Acanthamoeba sp. KA/E5 18S ribosomal RNA gene, complete sequence	744	744	97%	0.000E+00	99%
				AY173014.1	Acanthamoeba sp. KA/MSS6 18S ribosomal RNA gene, complete sequence	744	744	97%	0.000E+00	99%
				AY173013.1	Acanthamoeba sp. KA/MSS2 18S ribosomal RNA gene, complete sequence	744	744	97%	0.000E+00	99%
				AY026243.1	**Acanthamoeba polyphaga strain ATCC30461 small subunit ribosomal RNA gene, complete sequence**	**744**	**744**	**97%**	**0.000E+00**	**99%**

				JN222978.1	Acanthamoeba sp. 283 18S ribosomal RNA gene, partial sequence	742	742	97%	0.000E+00	99%
				DQ087320.1	Acanthamoeba sp. S4 18S ribosomal RNA gene, partial sequence	742	742	97%	0.000E+00	99%
7	Water	E_i2	*Acanthamoeba polyphaga*	**GU596994.1**	**Acanthamoeba polyphaga isolate A8/SB2 18S ribosomal RNA gene, partial sequence**	**740**	**740**	**98%**	**0.000E+00**	**99%**
				JN222978.1	Acanthamoeba sp. 283 18S ribosomal RNA gene, partial sequence	733	733	98%	0.000E+00	99%
				DQ087320.1	Acanthamoeba sp. S4 18S ribosomal RNA gene, partial sequence	733	733	98%	0.000E+00	99%
				AY148954.1	Acanthamoeba sp. KA/E5 18S ribosomal RNA gene, complete sequence	733	733	98%	0.000E+00	99%
				AY173014.1	Acanthamoeba sp. KA/MSS6 18S ribosomal RNA gene, complete sequence	733	733	98%	0.000E+00	99%

				AY173013.1	Acanthamoeba sp. KA/MSS2 18S ribosomal RNA gene, complete sequence	733	733	98%	0.000E+00	99%
				AY026243.1	Acanthamoeba polyphaga strain ATCC30461 small subunit ribosomal RNA gene, complete sequence	733	733	98%	0.000E+00	99%
8	Water	E_i3	*Acanthamoeba polyphaga*	AY148954.1	Acanthamoeba sp. KA/E5 18S ribosomal RNA gene, complete sequence	744	744	100%	0.000E+00	99%
				AY173014.1	Acanthamoeba sp. KA/MSS6 18S ribosomal RNA gene, complete sequence	744	744	100%	0.000E+00	99%
				AY173013.1	Acanthamoeba sp. KA/MSS2 18S ribosomal RNA gene, complete sequence	744	744	100%	0.000E+00	99%
				AY026243.1	**Acanthamoeba polyphaga strain ATCC30461 small subunit ribosomal RNA gene, complete sequence**	**744**	**744**	**100%**	**0.000E+00**	**99%**

				JN222978.1	Acanthamoeba sp. 283 18S ribosomal RNA gene, partial sequence	742	742	99%	0.000E+00	99%
				DQ087320.1	Acanthamoeba sp. S4 18S ribosomal RNA gene, partial sequence	742	742	99%	0.000E+00	99%
9	Water	E_i4	*Acanthamoeba polyphaga*	AY148954.1	Acanthamoeba sp. KA/E5 18S ribosomal RNA gene, complete sequence	747	747	97%	0.000E+00	99%
				AY173014.1	Acanthamoeba sp. KA/MSS6 18S ribosomal RNA gene, complete sequence	747	747	97%	0.000E+00	99%
				AY173013.1	Acanthamoeba sp. KA/MSS2 18S ribosomal RNA gene, complete sequence	747	747	97%	0.000E+00	99%
				AY026243.1	**Acanthamoeba polyphaga strain ATCC30461 small subunit ribosomal RNA gene, complete sequence**	**747**	**747**	**97%**	**0.000E+00**	**99%**
				JN222978.1	Acanthamoeba sp. 283 18S ribosomal RNA gene, partial sequence	746	746	97%	0.000E+00	99%

				DQ087320.1	Acanthamoeba sp. S4 18S ribosomal RNA gene, partial sequence	746	746	97%	0.000E+00	99%
10	Water	E_i5	*Acanthamoeba polyphaga*	AY148954.1	Acanthamoeba sp. KA/E5 18S ribosomal RNA gene, complete sequence	749	749	97%	0.000E+00	99%
				AY173014.1	Acanthamoeba sp. KA/MSS6 18S ribosomal RNA gene, complete sequence	749	749	97%	0.000E+00	99%
				AY173013.1	Acanthamoeba sp. KA/MSS2 18S ribosomal RNA gene, complete sequence	749	749	97%	0.000E+00	99%
				AY026243.1	**Acanthamoeba polyphaga strain ATCC30461 small subunit ribosomal RNA gene, complete sequence**	**749**	**749**	**97%**	**0.000E+00**	**99%**
				JN222978.1	Acanthamoeba sp. 283 18S ribosomal RNA gene, partial sequence	746	746	97%	0.000E+00	99%
				GQ397478.1	Acanthamoeba sp. AcaVN16 18S ribosomal RNA gene, partial sequence	746	746	97%	0.000E+00	99%

				EF140630.1	Acanthamoeba sp. KA/E29 18S ribosomal RNA gene, complete sequence	746	746	97%	0.000E+00	99%
				DQ087320.1	Acanthamoeba sp. S4 18S ribosomal RNA gene, partial sequence	746	746	97%	0.000E+00	99%
				DQ013363.1	Acanthamoeba polyphaga 18S ribosomal RNA gene, partial sequence	746	746	97%	0.000E+00	99%
				AY173004.1	Acanthamoeba sp. KA/MSG4 18S ribosomal RNA gene, complete sequence	746	746	97%	0.000E+00	99%
				U07407.1	Acanthamoeba polyphaga BCM:0173:16 ATCC 50371 18S rRNA gene	746	746	97%	0.000E+00	99%
11	Water	E_i6	*Acanthamoeba polyphaga*	**GU596994.1**	**Acanthamoeba polyphaga isolate A8/SB2 18S ribosomal RNA gene, partial sequence**	**737**	**737**	**96%**	**0.000E+00**	**99%**
				AY148954.1	Acanthamoeba sp. KA/E5 18S ribosomal RNA gene, complete sequence	733	733	96%	0.000E+00	99%

				AY173014.1	Acanthamoeba sp. KA/MSS6 18S ribosomal RNA gene, complete sequence	733	733	96%	0.000E+00	99%
				AY173013.1	Acanthamoeba sp. KA/MSS2 18S ribosomal RNA gene, complete sequence	733	733	96%	0.000E+00	99%
				AY026243.1	Acanthamoeba polyphaga strain ATCC30461 small subunit ribosomal RNA gene, complete sequence	733	733	96%	0.000E+00	99%
12	Water	E_i7	*Acanthamoeba polyphaga*	AY148954.1	Acanthamoeba sp. KA/E5 18S ribosomal RNA gene, complete sequence	746	746	97%	0.000E+00	99%
				AY173014.1	Acanthamoeba sp. KA/MSS6 18S ribosomal RNA gene, complete sequence	746	746	97%	0.000E+00	99%
				AY173013.1	Acanthamoeba sp. KA/MSS2 18S ribosomal RNA gene, complete sequence	746	746	97%	0.000E+00	99%

				AY026243.1	**Acanthamoeba polyphaga strain ATCC30461 small subunit ribosomal RNA gene, complete sequence**	**746**	**746**	**97%**	**0.000E+00**	**99%**
				JN222978.1	Acanthamoeba sp. 283 18S ribosomal RNA gene, partial sequence	742	742	96%	0.000E+00	99%
				DQ087320.1	Acanthamoeba sp. S4 18S ribosomal RNA gene, partial sequence	742	742	96%	0.000E+00	99%
				U07407.1	Acanthamoeba polyphaga BCM:0173:16 ATCC 50371 18S rRNA gene	742	742	97%	0.000E+00	99%
13	Water	E_i8	*Acanthamoeba polyphaga*	AY148954.1	Acanthamoeba sp. KA/E5 18S ribosomal RNA gene, complete sequence	751	751	98%	0.000E+00	99%
				AY173014.1	Acanthamoeba sp. KA/MSS6 18S ribosomal RNA gene, complete sequence	751	751	98%	0.000E+00	99%
				AY173013.1	Acanthamoeba sp. KA/MSS2 18S ribosomal RNA gene, complete sequence	751	751	98%	0.000E+00	99%

				AY026243.1	**Acanthamoeba polyphaga strain ATCC30461 small subunit ribosomal RNA gene, complete sequence**	**751**	**751**	**98%**	**0.000E+00**	**99%**
				JN222978.1	Acanthamoeba sp. 283 18S ribosomal RNA gene, partial sequence	749	749	98%	0.000E+00	99%
				DQ087320.1	Acanthamoeba sp. S4 18S ribosomal RNA gene, partial sequence	749	749	98%	0.000E+00	99%
14	Water	F_i1	*Acanthamoeba polyphaga*	AY148954.1	Acanthamoeba sp. KA/E5 18S ribosomal RNA gene, complete sequence	746	746	97%	0.000E+00	99%
				AY173014.1	Acanthamoeba sp. KA/MSS6 18S ribosomal RNA gene, complete sequence	746	746	97%	0.000E+00	99%
				AY173013.1	Acanthamoeba sp. KA/MSS2 18S ribosomal RNA gene, complete sequence	746	746	97%	0.000E+00	99%
				AY026243.1	**Acanthamoeba polyphaga strain ATCC30461 small subunit ribosomal RNA gene, complete sequence**	**746**	**746**	**97%**	**0.000E+00**	**99%**

				JN222978.1	Acanthamoeba sp. 283 18S ribosomal RNA gene, partial sequence	744	744	97%	0.000E+00	99%
				DQ087320.1	Acanthamoeba sp. S4 18S ribosomal RNA gene, partial sequence	744	744	97%	0.000E+00	99%
15	Water	F_i2	*Acanthamoeba polyphaga*	AY148954.1	Acanthamoeba sp. KA/E5 18S ribosomal RNA gene, complete sequence	744	744	100%	0.000E+00	99%
				AY173014.1	Acanthamoeba sp. KA/MSS6 18S ribosomal RNA gene, complete sequence	744	744	100%	0.000E+00	99%
				AY173013.1	Acanthamoeba sp. KA/MSS2 18S ribosomal RNA gene, complete sequence	744	744	100%	0.000E+00	99%
				AY026243.1	**Acanthamoeba polyphaga strain ATCC30461 small subunit ribosomal RNA gene, complete sequence**	**744**	**744**	**100%**	**0.000E+00**	**99%**
				JN222978.1	Acanthamoeba sp. 283 18S ribosomal RNA gene, partial sequence	742	742	99%	0.000E+00	99%

				DQ087320.1	Acanthamoeba sp. S4 18S ribosomal RNA gene, partial sequence	742	742	99%	0.000E+00	99%
16	Water	F_i3	*Acanthamoeba polyphaga*	AY148954.1	Acanthamoeba sp. KA/E5 18S ribosomal RNA gene, complete sequence	747	747	99%	0.000E+00	99%
				AY173014.1	Acanthamoeba sp. KA/MSS6 18S ribosomal RNA gene, complete sequence	747	747	99%	0.000E+00	99%
				AY173013.1	Acanthamoeba sp. KA/MSS2 18S ribosomal RNA gene, complete sequence	747	747	99%	0.000E+00	99%
				AY026243.1	**Acanthamoeba polyphaga strain ATCC30461 small subunit ribosomal RNA gene, complete sequence**	**747**	**747**	**99%**	**0.000E+00**	**99%**
				JN222978.1	Acanthamoeba sp. 283 18S ribosomal RNA gene, partial sequence	744	744	98%	0.000E+00	99%
				DQ087320.1	Acanthamoeba sp. S4 18S ribosomal RNA gene, partial sequence	744	744	98%	0.000E+00	99%

				U07407.1	Acanthamoeba polyphaga BCM:0173:16 ATCC 50371 18S rRNA gene	744	744	99%	0.000E+00	99%
17	Water	B_i1	*Acanthamoeba polyphaga*	AY148954.1	Acanthamoeba sp. KA/E5 18S ribosomal RNA gene, complete sequence	746	746	97%	0.000E+00	99%
				AY173014.1	Acanthamoeba sp. KA/MSS6 18S ribosomal RNA gene, complete sequence	746	746	97%	0.000E+00	99%
				AY173013.1	Acanthamoeba sp. KA/MSS2 18S ribosomal RNA gene, complete sequence	746	746	97%	0.000E+00	99%
				AY026243.1	**Acanthamoeba polyphaga strain ATCC30461 small subunit ribosomal RNA gene, complete sequence**	**746**	**746**	**97%**	**0.000E+00**	**99%**
				JN222978.1	Acanthamoeba sp. 283 18S ribosomal RNA gene, partial sequence	742	742	96%	0.000E+00	99%
				DQ087320.1	Acanthamoeba sp. S4 18S ribosomal RNA gene, partial sequence	742	742	96%	0.000E+00	99%

				U07407.1	Acanthamoeba polyphaga BCM:0173:16 ATCC 50371 18S rRNA gene	742	742	97%	0.000E+00	99%
18	Water	B_i2	*Acanthamoeba polyphaga*	AY148954.1	Acanthamoeba sp. KA/E5 18S ribosomal RNA gene, complete sequence	746	746	97%	0.000E+00	99%
				AY173014.1	Acanthamoeba sp. KA/MSS6 18S ribosomal RNA gene, complete sequence	746	746	97%	0.000E+00	99%
				AY173013.1	Acanthamoeba sp. KA/MSS2 18S ribosomal RNA gene, complete sequence	746	746	97%	0.000E+00	99%
				AY026243.1	**Acanthamoeba polyphaga strain ATCC30461 small subunit ribosomal RNA gene, complete sequence**	**746**	**746**	**97%**	**0.000E+00**	**99%**
				JN222978.1	Acanthamoeba sp. 283 18S ribosomal RNA gene, partial sequence	742	742	97%	0.000E+00	99%
				DQ087320.1	Acanthamoeba sp. S4 18S ribosomal RNA gene, partial sequence	742	742	97%	0.000E+00	99%

				U07407.1	Acanthamoeba polyphaga BCM:0173:16 ATCC 50371 18S rRNA gene	742	742	97%	0.000E+00	99%
19	Water	B_i3	*Acanthamoeba polyphaga*	**GU596994.1**	**Acanthamoeba polyphaga isolate A8/SB2 18S ribosomal RNA gene, partial sequence**	**740**	**740**	**98%**	**0.000E+00**	**99%**
				JN222978.1	Acanthamoeba sp. 283 18S ribosomal RNA gene, partial sequence	733	733	98%	0.000E+00	99%
				DQ087320.1	Acanthamoeba sp. S4 18S ribosomal RNA gene, partial sequence	733	733	98%	0.000E+00	99%
				AY148954.1	Acanthamoeba sp. KA/E5 18S ribosomal RNA gene, complete sequence	733	733	98%	0.000E+00	99%
				AY173014.1	Acanthamoeba sp. KA/MSS6 18S ribosomal RNA gene, complete sequence	733	733	98%	0.000E+00	99%
				AY173013.1	Acanthamoeba sp. KA/MSS2 18S ribosomal RNA gene, complete sequence	733	733	98%	0.000E+00	99%

				AY026243.1	Acanthamoeba polyphaga strain ATCC30461 small subunit ribosomal RNA gene, complete sequence	733	733	98%	0.000E+00	99%
20	Water	B_i4	*Acanthamoeba polyphaga*	**GU596994.1**	**Acanthamoeba polyphaga isolate A8/SB2 18S ribosomal RNA gene, partial sequence**	**742**	**742**	**96%**	**0.000E+00**	**99%**
				AY148954.1	Acanthamoeba sp. KA/E5 18S ribosomal RNA gene, complete sequence	738	738	99%	0.000E+00	98%
				AY173014.1	Acanthamoeba sp. KA/MSS6 18S ribosomal RNA gene, complete sequence	738	738	99%	0.000E+00	98%
				AY173013.1	Acanthamoeba sp. KA/MSS2 18S ribosomal RNA gene, complete sequence	738	738	99%	0.000E+00	98%
				AY026243.1	Acanthamoeba polyphaga strain ATCC30461 small subunit ribosomal RNA gene, complete sequence	738	738	99%	0.000E+00	98%

21	Water	B_i5	*Acanthamoeba polyphaga*	**GU596994.1**	**Acanthamoeba polyphaga isolate A8/SB2 18S ribosomal RNA gene, partial sequence**	**737**	**737**	**96%**	**0.000E+00**	**99%**
				AY148954.1	Acanthamoeba sp. KA/E5 18S ribosomal RNA gene, complete sequence	733	733	96%	0.000E+00	99%
				AY173014.1	Acanthamoeba sp. KA/MSS6 18S ribosomal RNA gene, complete sequence	733	733	96%	0.000E+00	99%
				AY173013.1	Acanthamoeba sp. KA/MSS2 18S ribosomal RNA gene, complete sequence	733	733	96%	0.000E+00	99%
				AY026243.1	Acanthamoeba polyphaga strain ATCC30461 small subunit ribosomal RNA gene, complete sequence	733	733	96%	0.000E+00	99%
22	Water	C_i1	*Acanthamoeba polyphaga*	AY148954.1	Acanthamoeba sp. KA/E5 18S ribosomal RNA gene, complete sequence	749	749	97%	0.000E+00	99%
				AY173014.1	Acanthamoeba sp. KA/MSS6 18S ribosomal RNA gene, complete	749	749	97%	0.000E+00	99%

	sequence					
AY173013.1	Acanthamoeba sp. KA/MSS2 18S ribosomal RNA gene, complete sequence	749	749	97%	0.000E+00	99%
AY026243.1	**Acanthamoeba polyphaga strain ATCC30461 small subunit ribosomal RNA gene, complete sequence**	**749**	**749**	**97%**	**0.000E+00**	**99%**
JN222978.1	Acanthamoeba sp. 283 18S ribosomal RNA gene, partial sequence	746	746	97%	0.000E+00	99%
GQ397478.1	Acanthamoeba sp. AcaVN16 18S ribosomal RNA gene, partial sequence	746	746	97%	0.000E+00	99%
EF140630.1	Acanthamoeba sp. KA/E29 18S ribosomal RNA gene, complete sequence	746	746	97%	0.000E+00	99%
DQ087320.1	Acanthamoeba sp. S4 18S ribosomal RNA gene, partial sequence	746	746	97%	0.000E+00	99%
DQ013363.1	Acanthamoeba polyphaga 18S ribosomal RNA gene, partial sequence	746	746	97%	0.000E+00	99%

				AY173004.1	Acanthamoeba sp. KA/MSG4 18S ribosomal RNA gene, complete sequence	746	746	97%	0.000E+00	99%
				U07407.1	Acanthamoeba polyphaga BCM:0173:16 ATCC 50371 18S rRNA gene	746	746	97%	0.000E+00	99%
23	Water	C_i2	*Acanthamoeba polyphaga*	AY148954.1	Acanthamoeba sp. KA/E5 18S ribosomal RNA gene, complete sequence	747	747	97%	0.000E+00	99%
				AY173014.1	Acanthamoeba sp. KA/MSS6 18S ribosomal RNA gene, complete sequence	747	747	97%	0.000E+00	99%
				AY173013.1	Acanthamoeba sp. KA/MSS2 18S ribosomal RNA gene, complete sequence	747	747	97%	0.000E+00	99%
				AY026243.1	**Acanthamoeba polyphaga strain ATCC30461 small subunit ribosomal RNA gene, complete sequence**	**747**	**747**	**97%**	**0.000E+00**	**99%**
				JN222978.1	Acanthamoeba sp. 283 18S ribosomal RNA gene, partial sequence	746	746	97%	0.000E+00	99%

				DQ087320.1	Acanthamoeba sp. S4 18S ribosomal RNA gene, partial sequence	746	746	97%	0.000E+00	99%
24	Water	C_i3	*Acanthamoeba polyphaga*	AY148954.1	Acanthamoeba sp. KA/E5 18S ribosomal RNA gene, complete sequence	751	751	98%	0.000E+00	99%
				AY173014.1	Acanthamoeba sp. KA/MSS6 18S ribosomal RNA gene, complete sequence	751	751	98%	0.000E+00	99%
				AY173013.1	Acanthamoeba sp. KA/MSS2 18S ribosomal RNA gene, complete sequence	751	751	98%	0.000E+00	99%
				AY026243.1	**Acanthamoeba polyphaga strain ATCC30461 small subunit ribosomal RNA gene, complete sequence**	**751**	**751**	**98%**	**0.000E+00**	**99%**
				JN222978.1	Acanthamoeba sp. 283 18S ribosomal RNA gene, partial sequence	749	749	98%	0.000E+00	99%
				DQ087320.1	Acanthamoeba sp. S4 18S ribosomal RNA gene, partial sequence	749	749	98%	0.000E+00	99%

25	Water	D_i1	*Acanthamoeba polyphaga*	AY148954.1	Acanthamoeba sp. KA/E5 18S ribosomal RNA gene, complete sequence	747	747	99%	0.000E+00	99%
				AY173014.1	Acanthamoeba sp. KA/MSS6 18S ribosomal RNA gene, complete sequence	747	747	99%	0.000E+00	99%
				AY173013.1	Acanthamoeba sp. KA/MSS2 18S ribosomal RNA gene, complete sequence	747	747	99%	0.000E+00	99%
				AY026243.1	**Acanthamoeba polyphaga strain ATCC30461 small subunit ribosomal RNA gene, complete sequence**	**747**	**747**	**99%**	**0.000E+00**	**99%**
				JN222978.1	Acanthamoeba sp. 283 18S ribosomal RNA gene, partial sequence	744	744	98%	0.000E+00	99%
				DQ087320.1	Acanthamoeba sp. S4 18S ribosomal RNA gene, partial sequence	744	744	98%	0.000E+00	99%
				U07407.1	Acanthamoeba polyphaga BCM:0173:16 ATCC 50371 18S rRNA gene	744	744	99%	0.000E+00	99%

26	Water	D_i2	*Acanthamoeba polyphaga*	AY148954.1	Acanthamoeba sp. KA/E5 18S ribosomal RNA gene, complete sequence	746	746	97%	0.000E+00	99%
				AY173014.1	Acanthamoeba sp. KA/MSS6 18S ribosomal RNA gene, complete sequence	746	746	97%	0.000E+00	99%
				AY173013.1	Acanthamoeba sp. KA/MSS2 18S ribosomal RNA gene, complete sequence	746	746	97%	0.000E+00	99%
				AY026243.1	**Acanthamoeba polyphaga strain ATCC30461 small subunit ribosomal RNA gene, complete sequence**	**746**	**746**	**97%**	**0.000E+00**	**99%**
				JN222978.1	Acanthamoeba sp. 283 18S ribosomal RNA gene, partial sequence	744	744	97%	0.000E+00	99%
				DQ087320.1	Acanthamoeba sp. S4 18S ribosomal RNA gene, partial sequence	744	744	97%	0.000E+00	99%
27	Soil	HC1_Soil_s2	*Acanthamoeba polyphaga*	**GU596995.1**	**Acanthamoeba polyphaga isolate A10/SB2 18S ribosomal RNA gene, partial sequence**	**735**	**735**	**97%**	**0.000E+00**	**99%**

				AY148954.1	Acanthamoeba sp. KA/E5 18S ribosomal RNA gene, complete sequence	728	728	97%	0.000E+00	98%
				AY173014.1	Acanthamoeba sp. KA/MSS6 18S ribosomal RNA gene, complete sequence	728	728	97%	0.000E+00	98%
				AY173013.1	Acanthamoeba sp. KA/MSS2 18S ribosomal RNA gene, complete sequence	728	728	97%	0.000E+00	98%
				AY026243.1	Acanthamoeba polyphaga strain ATCC30461 small subunit ribosomal RNA gene, complete sequence	728	728	97%	0.000E+00	98%
28	Soil	HC1_Soil_s5	*Acanthamoeba lenticulata*	**U94741.1**	**Acanthamoeba lenticulata strain PD2S 18S small subunit ribosomal RNA gene, partial sequence**	**690**	**690**	**98%**	**0.000E+00**	**99%**
				U94740.1	**Acanthamoeba lenticulata strain 25/1 18S small subunit ribosomal RNA gene, partial sequence**	**690**	**690**	**98%**	**0.000E+00**	**99%**

U94738.1	**Acanthamoeba lenticulata strain NJSP-3-2 18S small subunit ribosomal RNA gene, partial sequence**	**690**	**690**	**98%**	**0.000E+00**	**99%**
U94737.1	**Acanthamoeba lenticulata strain 53-2 18S small subunit ribosomal RNA gene, partial sequence**	**690**	**690**	**98%**	**0.000E+00**	**99%**
U94736.1	**Acanthamoeba lenticulata strain 118 18S small subunit ribosomal RNA gene, partial sequence**	**690**	**690**	**98%**	**0.000E+00**	**99%**
U94735.1	**Acanthamoeba lenticulata strain E18-2 18S small subunit ribosomal RNA gene, partial sequence**	**690**	**690**	**98%**	**0.000E+00**	**99%**
U94734.1	**Acanthamoeba lenticulata strain 407-3a 18S small subunit ribosomal RNA gene, partial sequence**	**690**	**690**	**98%**	**0.000E+00**	**99%**
U94733.1	**Acanthamoeba lenticulata strain 68-2 18S small subunit ribosomal RNA gene, partial sequence**	**690**	**690**	**98%**	**0.000E+00**	**99%**

				AB525818.1	Acanthamoeba sp. AH-2009 gene for 18S ribosomal RNA, partial sequence, strain: NHE(Ac)1-IR-T5	686	686	98%	0.000E+00	99%
				EU146072.1	Acanthamoeba sp. S35 18S ribosomal RNA gene, partial sequence	686	686	98%	0.000E+00	99%
29	Soil	CC1_Soil_s1	*Acanthamoeba lenticulata*	**U94741.1**	**Acanthamoeba lenticulata strain PD2S 18S small subunit ribosomal RNA gene, partial sequence**	**688**	**688**	**96%**	**0.000E+00**	**99%**
				U94740.1	**Acanthamoeba lenticulata strain 25/1 18S small subunit ribosomal RNA gene, partial sequence**	**688**	**688**	**96%**	**0.000E+00**	**99%**
				U94738.1	**Acanthamoeba lenticulata strain NJSP-3-2 18S small subunit ribosomal RNA gene, partial sequence**	**688**	**688**	**96%**	**0.000E+00**	**99%**
				U94737.1	**Acanthamoeba lenticulata strain 53-2 18S small subunit ribosomal RNA gene, partial sequence**	**688**	**688**	**96%**	**0.000E+00**	**99%**

U94736.1	**Acanthamoeba lenticulata strain 118 18S small subunit ribosomal RNA gene, partial sequence**	**688**	**688**	**96%**	**0.000E+00**	**99%**
U94735.1	**Acanthamoeba lenticulata strain E18-2 18S small subunit ribosomal RNA gene, partial sequence**	**688**	**688**	**96%**	**0.000E+00**	**99%**
U94734.1	**Acanthamoeba lenticulata strain 407-3a 18S small subunit ribosomal RNA gene, partial sequence**	**688**	**688**	**96%**	**0.000E+00**	**99%**
U94733.1	**Acanthamoeba lenticulata strain 68-2 18S small subunit ribosomal RNA gene, partial sequence**	**688**	**688**	**96%**	**0.000E+00**	**99%**
AB525818.1	Acanthamoeba sp. AH-2009 gene for 18S ribosomal RNA, partial sequence, strain: NHE(Ac)1-IR-T5	684	684	96%	0.000E+00	99%
EU146072.1	Acanthamoeba sp. S35 18S ribosomal RNA gene, partial sequence	684	684	96%	0.000E+00	99%

30	Soil	CC1_Soil_s5	*Acanthamoeba lenticulata*	**U94741.1**	**Acanthamoeba lenticulata strain PD2S 18S small subunit ribosomal RNA gene, partial sequence**	**688**	**688**	**96%**	**0.000E+00**	**99%**
				U94740.1	**Acanthamoeba lenticulata strain 25/1 18S small subunit ribosomal RNA gene, partial sequence**	**688**	**688**	**96%**	**0.000E+00**	**99%**
				U94738.1	**Acanthamoeba lenticulata strain NJSP-3-2 18S small subunit ribosomal RNA gene, partial sequence**	**688**	**688**	**96%**	**0.000E+00**	**99%**
				U94737.1	**Acanthamoeba lenticulata strain 53-2 18S small subunit ribosomal RNA gene, partial sequence**	**688**	**688**	**96%**	**0.000E+00**	**99%**
				U94736.1	**Acanthamoeba lenticulata strain 118 18S small subunit ribosomal RNA gene, partial sequence**	**688**	**688**	**96%**	**0.000E+00**	**99%**
				U94735.1	**Acanthamoeba lenticulata strain E18-2 18S small subunit ribosomal RNA gene, partial sequence**	**688**	**688**	**96%**	**0.000E+00**	**99%**

				U94734.1	**Acanthamoeba lenticulata strain 407-3a 18S small subunit ribosomal RNA gene, partial sequence**	**688**	**688**	**96%**	**0.000E+00**	**99%**
				U94733.1	**Acanthamoeba lenticulata strain 68-2 18S small subunit ribosomal RNA gene, partial sequence**	**688**	**688**	**96%**	**0.000E+00**	**99%**
				AB525818.1	Acanthamoeba sp. AH-2009 gene for 18S ribosomal RNA, partial sequence, strain: NHE(Ac)1-IR-T5	684	684	96%	0.000E+00	99%
				EU146072.1	Acanthamoeba sp. S35 18S ribosomal RNA gene, partial sequence	684	684	96%	0.000E+00	99%
31	Soil	HC2_Soil_s1	*Acanthamoeba polyphaga*	JQ031557.1	Acanthamoeba sp. AcaKM01 18S ribosomal RNA gene, partial sequence	713	713	97%	0.000E+00	98%
				GU936484.1	Acanthamoeba sp. M3 YH-2010 18S ribosomal RNA gene, partial sequence	711	711	97%	0.000E+00	98%
				DQ992185.1	Acanthamoeba sp. EFW4 18S ribosomal RNA gene, partial sequence	711	711	97%	0.000E+00	98%

DQ992184.1	Acanthamoeba sp. EFW2 18S ribosomal RNA gene, partial sequence	711	711	97%	0.000E+00	98%
DQ992183.1	Acanthamoeba sp. EFW8 18S ribosomal RNA gene, partial sequence	711	711	97%	0.000E+00	98%
DQ992182.1	Acanthamoeba sp. EFW6 18S ribosomal RNA gene, partial sequence	711	711	97%	0.000E+00	98%
DQ103890.1	Acanthamoeba sp. MZOR 18S ribosomal RNA gene, partial sequence	711	711	97%	0.000E+00	98%
AY694141.1	Acanthamoeba sp. L749/98 18S ribosomal RNA gene, complete sequence	711	711	97%	0.000E+00	98%
AY148956.1	Acanthamoeba sp. KA/E8 18S ribosomal RNA gene, complete sequence	711	711	97%	0.000E+00	98%
AY026749.1	Acanthamoeba sp. isolate U/E10 small subunit ribosomal RNA gene, complete sequence	711	711	97%	0.000E+00	98%
AF019062.1	**Acanthamoeba polyphaga Nagington 18S ribosomal RNA gene, partial**	**711**	**711**	**97%**	**0.000E+00**	**98%**

					sequence					
32	Soil	HC2_Soil_s3	*Acanthamoeba polyphaga*	**GU596995.1**	**Acanthamoeba polyphaga isolate A10/SB2 18S ribosomal RNA gene, partial sequence**	**735**	**735**	**97%**	**0.000E+00**	**99%**
				AY148954.1	Acanthamoeba sp. KA/E5 18S ribosomal RNA gene, complete sequence	728	728	97%	0.000E+00	98%
				AY173014.1	Acanthamoeba sp. KA/MSS6 18S ribosomal RNA gene, complete sequence	728	728	97%	0.000E+00	98%
				AY173013.1	Acanthamoeba sp. KA/MSS2 18S ribosomal RNA gene, complete sequence	728	728	97%	0.000E+00	98%
				AY026243.1	Acanthamoeba polyphaga strain ATCC30461 small subunit ribosomal RNA gene, complete sequence	728	728	97%	0.000E+00	98%
33	Soil	HC2_Soil_s4	*Acanthamoeba lenticulata*	**U94741.1**	**Acanthamoeba lenticulata strain PD2S 18S small subunit ribosomal RNA**	**688**	**688**	**97%**	**0.000E+00**	**99%**

	gene, partial sequence					
U94740.1	Acanthamoeba lenticulata strain 25/1 18S small subunit ribosomal RNA gene, partial sequence	688	688	97%	0.000E+00	99%
U94738.1	Acanthamoeba lenticulata strain NJSP-3-2 18S small subunit ribosomal RNA gene, partial sequence	688	688	97%	0.000E+00	99%
U94737.1	Acanthamoeba lenticulata strain 53-2 18S small subunit ribosomal RNA gene, partial sequence	688	688	97%	0.000E+00	99%
U94736.1	Acanthamoeba lenticulata strain 118 18S small subunit ribosomal RNA gene, partial sequence	688	688	97%	0.000E+00	99%
U94735.1	Acanthamoeba lenticulata strain E18-2 18S small subunit ribosomal RNA gene, partial sequence	688	688	97%	0.000E+00	99%
U94734.1	Acanthamoeba lenticulata strain 407-3a 18S small	688	688	97%	0.000E+00	99%

					subunit ribosomal RNA gene, partial sequence					
				U94733.1	**Acanthamoeba lenticulata strain 68-2 18S small subunit ribosomal RNA gene, partial sequence**	**688**	**688**	**97%**	**0.000E+00**	**99%**
				AB525818.1	Acanthamoeba sp. AH-2009 gene for 18S ribosomal RNA, partial sequence, strain: NHE(Ac)1-IR-T5	684	684	96%	0.000E+00	99%
				EU146072.1	Acanthamoeba sp. S35 18S ribosomal RNA gene, partial sequence	684	684	96%	0.000E+00	99%
34	Soil	HC2_Soil_s5	*Acanthamoeba lenticulata*	**U94741.1**	**Acanthamoeba lenticulata strain PD2S 18S small subunit ribosomal RNA gene, partial sequence**	**688**	**688**	**96%**	**0.000E+00**	**99%**
				U94740.1	**Acanthamoeba lenticulata strain 25/1 18S small subunit ribosomal RNA gene, partial sequence**	**688**	**688**	**96%**	**0.000E+00**	**99%**
				U94738.1	**Acanthamoeba lenticulata strain NJSP-3-2 18S small subunit ribosomal RNA**	**688**	**688**	**96%**	**0.000E+00**	**99%**

	gene, partial sequence					
U94737.1	**Acanthamoeba lenticulata strain 53-2 18S small subunit ribosomal RNA gene, partial sequence**	**688**	**688**	**96%**	**0.000E+00**	**99%**
U94736.1	**Acanthamoeba lenticulata strain 118 18S small subunit ribosomal RNA gene, partial sequence**	**688**	**688**	**96%**	**0.000E+00**	**99%**
U94735.1	**Acanthamoeba lenticulata strain E18-2 18S small subunit ribosomal RNA gene, partial sequence**	**688**	**688**	**96%**	**0.000E+00**	**99%**
U94734.1	**Acanthamoeba lenticulata strain 407-3a 18S small subunit ribosomal RNA gene, partial sequence**	**688**	**688**	**96%**	**0.000E+00**	**99%**
U94733.1	**Acanthamoeba lenticulata strain 68-2 18S small subunit ribosomal RNA gene, partial sequence**	**688**	**688**	**96%**	**0.000E+00**	**99%**
AB525818.1	Acanthamoeba sp. AH-2009 gene for 18S ribosomal	684	684	96%	0.000E+00	99%

					RNA, partial sequence, strain: NHE(Ac)1-IR-T5					
				EU146072.1	Acanthamoeba sp. S35 18S ribosomal RNA gene, partial sequence	684	684	96%	0.000E+00	99%
35	Soil	HC2_Soil_s6	*Acanthamoeba polyphaga*	JQ031557.1	Acanthamoeba sp. AcaKM01 18S ribosomal RNA gene, partial sequence	713	713	97%	0.000E+00	98%
				GU936484.1	Acanthamoeba sp. M3 YH-2010 18S ribosomal RNA gene, partial sequence	711	711	97%	0.000E+00	98%
				DQ992185.1	Acanthamoeba sp. EFW4 18S ribosomal RNA gene, partial sequence	711	711	97%	0.000E+00	98%
				DQ992184.1	Acanthamoeba sp. EFW2 18S ribosomal RNA gene, partial sequence	711	711	97%	0.000E+00	98%
				DQ992183.1	Acanthamoeba sp. EFW8 18S ribosomal RNA gene, partial sequence	711	711	97%	0.000E+00	98%
				DQ992182.1	Acanthamoeba sp. EFW6 18S ribosomal RNA gene, partial sequence	711	711	97%	0.000E+00	98%

				DQ103890.1	Acanthamoeba sp. MZOR 18S ribosomal RNA gene, partial sequence	711	711	97%	0.000E+00	98%
				AY694141.1	Acanthamoeba sp. L749/98 18S ribosomal RNA gene, complete sequence	711	711	97%	0.000E+00	98%
				AY148956.1	Acanthamoeba sp. KA/E8 18S ribosomal RNA gene, complete sequence	711	711	97%	0.000E+00	98%
				AY026749.1	Acanthamoeba sp. isolate U/E10 small subunit ribosomal RNA gene, complete sequence	711	711	97%	0.000E+00	98%
				AF019062.1	**Acanthamoeba polyphaga Nagington 18S ribosomal RNA gene, partial sequence**	**711**	**711**	**97%**	**0.000E+00**	**98%**
36	Soil	CC2_Soil_s1	*Acanthamoeba polyphaga*	AY148954.1	Acanthamoeba sp. KA/E5 18S ribosomal RNA gene, complete sequence	753	753	99%	0.000E+00	99%
				AY173014.1	Acanthamoeba sp. KA/MSS6 18S ribosomal RNA gene, complete sequence	753	753	99%	0.000E+00	99%

				AY173013.1	Acanthamoeba sp. KA/MSS2 18S ribosomal RNA gene, complete sequence	753	753	99%	0.000E+00	99%
				AY026243.1	**Acanthamoeba polyphaga strain ATCC30461 small subunit ribosomal RNA gene, complete sequence**	**753**	**753**	**99%**	**0.000E+00**	**99%**
				JN222978.1	Acanthamoeba sp. 283 18S ribosomal RNA gene, partial sequence	749	749	98%	0.000E+00	99%
				DQ087320.1	Acanthamoeba sp. S4 18S ribosomal RNA gene, partial sequence	749	749	98%	0.000E+00	99%
				U07407.1	Acanthamoeba polyphaga BCM:0173:16 ATCC 50371 18S rRNA gene	749	749	99%	0.000E+00	99%
37	Soil	CC2_Soil_s2	*Acanthamoeba polyphaga*	AY148954.1	Acanthamoeba sp. KA/E5 18S ribosomal RNA gene, complete sequence	749	749	97%	0.000E+00	99%
				AY173014.1	Acanthamoeba sp. KA/MSS6 18S ribosomal RNA gene, complete sequence	749	749	97%	0.000E+00	99%

				AY173013.1	Acanthamoeba sp. KA/MSS2 18S ribosomal RNA gene, complete sequence	749	749	97%	0.000E+00	99%
				AY026243.1	**Acanthamoeba polyphaga strain ATCC30461 small subunit ribosomal RNA gene, complete sequence**	**749**	**749**	**97%**	**0.000E+00**	**99%**
				JN222978.1	Acanthamoeba sp. 283 18S ribosomal RNA gene, partial sequence	747	747	97%	0.000E+00	99%
				DQ087320.1	Acanthamoeba sp. S4 18S ribosomal RNA gene, partial sequence	747	747	97%	0.000E+00	99%
38	Soil	CC2_Soil_s3	*Acanthamoeba lenticulata*	**U94741.1**	**Acanthamoeba lenticulata strain PD2S 18S small subunit ribosomal RNA gene, partial sequence**	**688**	**688**	**96%**	**0.000E+00**	**99%**
				U94740.1	**Acanthamoeba lenticulata strain 25/1 18S small subunit ribosomal RNA gene, partial sequence**	**688**	**688**	**96%**	**0.000E+00**	**99%**
				U94738.1	**Acanthamoeba lenticulata strain NJSP-3-2 18S small subunit ribosomal RNA**	**688**	**688**	**96%**	**0.000E+00**	**99%**

	gene, partial sequence					
U94737.1	**Acanthamoeba lenticulata strain 53-2 18S small subunit ribosomal RNA gene, partial sequence**	**688**	**688**	**96%**	**0.000E+00**	**99%**
U94736.1	**Acanthamoeba lenticulata strain 118 18S small subunit ribosomal RNA gene, partial sequence**	**688**	**688**	**96%**	**0.000E+00**	**99%**
U94735.1	**Acanthamoeba lenticulata strain E18-2 18S small subunit ribosomal RNA gene, partial sequence**	**688**	**688**	**96%**	**0.000E+00**	**99%**
U94734.1	**Acanthamoeba lenticulata strain 407-3a 18S small subunit ribosomal RNA gene, partial sequence**	**688**	**688**	**96%**	**0.000E+00**	**99%**
U94733.1	**Acanthamoeba lenticulata strain 68-2 18S small subunit ribosomal RNA gene, partial sequence**	**688**	**688**	**96%**	**0.000E+00**	**99%**
AB525818.1	Acanthamoeba sp. AH-2009 gene for 18S ribosomal	684	684	96%	0.000E+00	99%

					RNA, partial sequence, strain: NHE(Ac)1-IR-T5					
				EU146072.1	Acanthamoeba sp. S35 18S ribosomal RNA gene, partial sequence	684	684	96%	0.000E+00	99%
39	Soil	CC2_Soil_s4	*Acanthamoeba lenticulata*	**U94741.1**	**Acanthamoeba lenticulata strain PD2S 18S small subunit ribosomal RNA gene, partial sequence**	**690**	**690**	**98%**	**0.000E+00**	**99%**
				U94740.1	**Acanthamoeba lenticulata strain 25/1 18S small subunit ribosomal RNA gene, partial sequence**	**690**	**690**	**98%**	**0.000E+00**	**99%**
				U94738.1	**Acanthamoeba lenticulata strain NJSP-3-2 18S small subunit ribosomal RNA gene, partial sequence**	**690**	**690**	**98%**	**0.000E+00**	**99%**
				U94737.1	**Acanthamoeba lenticulata strain 53-2 18S small subunit ribosomal RNA gene, partial sequence**	**690**	**690**	**98%**	**0.000E+00**	**99%**
				U94736.1	**Acanthamoeba lenticulata strain 118 18S small subunit ribosomal RNA**	**690**	**690**	**98%**	**0.000E+00**	**99%**

					gene, partial sequence					
				U94735.1	**Acanthamoeba lenticulata strain E18-2 18S small subunit ribosomal RNA gene, partial sequence**	**690**	**690**	**98%**	**0.000E+00**	**99%**
				U94734.1	**Acanthamoeba lenticulata strain 407-3a 18S small subunit ribosomal RNA gene, partial sequence**	**690**	**690**	**98%**	**0.000E+00**	**99%**
				U94733.1	**Acanthamoeba lenticulata strain 68-2 18S small subunit ribosomal RNA gene, partial sequence**	**690**	**690**	**98%**	**0.000E+00**	**99%**
				AB525818.1	Acanthamoeba sp. AH-2009 gene for 18S ribosomal RNA, partial sequence, strain: NHE(Ac)1-IR-T5	686	686	98%	0.000E+00	99%
				EU146072.1	Acanthamoeba sp. S35 18S ribosomal RNA gene, partial sequence	686	686	98%	0.000E+00	99%
40	Air	HC1_Air_s4	*Acanthamoeba polyphaga*	AY148954.1	Acanthamoeba sp. KA/E5 18S ribosomal RNA gene, complete sequence	742	742	97%	0.000E+00	99%

				AY173014.1	Acanthamoeba sp. KA/MSS6 18S ribosomal RNA gene, complete sequence	742	742	97%	0.000E+00	99%
				AY173013.1	Acanthamoeba sp. KA/MSS2 18S ribosomal RNA gene, complete sequence	742	742	97%	0.000E+00	99%
				AY026243.1	**Acanthamoeba polyphaga strain ATCC30461 small subunit ribosomal RNA gene, complete sequence**	**742**	**742**	**97%**	**0.000E+00**	**99%**
				JN222978.1	Acanthamoeba sp. 283 18S ribosomal RNA gene, partial sequence	738	738	96%	0.000E+00	99%
				DQ087320.1	Acanthamoeba sp. S4 18S ribosomal RNA gene, partial sequence	738	738	96%	0.000E+00	99%
				U07407.1	Acanthamoeba polyphaga BCM:0173:16 ATCC 50371 18S rRNA gene	738	738	97%	0.000E+00	99%
41	Air	HC2_Air_s3	*Acanthamoeba jacobsi*	AY026245.1	Acanthamoeba sp. U/H-C1 small subunit ribosomal RNA gene, complete sequence	609	609	99%	8.000E-171	91%

				GQ380408.2	Acanthamoeba sp. cvX 18S ribosomal RNA gene, partial sequence	600	600	99%	4.000E-168	90%
				AY262364.1	**Acanthamoeba jacobsi AC304 18S ribosomal RNA gene, partial sequence**	**592**	**592**	**99%**	**6E-166**	**90%**
				AY262363.1	**Acanthamoeba jacobsi AC227 18S ribosomal RNA gene, partial sequence**	**592**	**592**	**99%**	**6E-166**	**90%**
				AY262362.1	**Acanthamoeba jacobsi AC194 18S ribosomal RNA gene, partial sequence**	**592**	**592**	**99%**	**6E-166**	**90%**
42	Air	HC2_Air_s6	*Acanthamoeba polyphaga*	**GU596994.1**	**Acanthamoeba polyphaga isolate A8/SB2 18S ribosomal RNA gene, partial sequence**	**746**	**746**	**96%**	**0.000E+00**	**99%**
				AY148954.1	Acanthamoeba sp. KA/E5 18S ribosomal RNA gene, complete sequence	744	744	98%	0	99%
				AY173014.1	Acanthamoeba sp. KA/MSS6 18S ribosomal RNA gene, complete sequence	744	744	98%	0	99%

AY173013.1	Acanthamoeba sp. KA/MSS2 18S ribosomal RNA gene, complete sequence	744	744	98%	0	99%
AY026243.1	Acanthamoeba polyphaga strain ATCC30461 small subunit ribosomal RNA gene, complete sequence	744	744	98%	0	99%

[a] Description in boldface represents the alignment and species to corresponding isolate

[b] Max score is the calculated from the number of matches and gaps and the higher relative to your query length is better

[c] Total score is the sum of the score of all (high scoring pairs, HSPs) from the same database sequence

[d] Query coverage is the percent of query sequence matched by database entry and is the length coverage of the input query sequence

[e] E value gives you the number of entries required in the database for a match to happen by random chance

f is the percent that the genes match up within the limits of the full match

Table 17 Grouping of 42 isolates based on accession number and query coverage value

Group	ASA.S1 sequences of 42 isolates	Published 18S rRNA sequences of *Acanthamoeba*		
		Accession	Description	Query coverage, %
1	E_i3 and F_i2	AY148954.1	Acanthamoeba sp. KA/E5 18S ribosomal RNA gene, complete sequence	100
		AY173014.1	Acanthamoeba sp. KA/MSS6 18S ribosomal RNA gene, complete sequence	100
		AY173013.1	Acanthamoeba sp. KA/MSS2 18S ribosomal RNA gene, complete sequence	100
		AY026243.1	Acanthamoeba polyphaga strain ATCC30461 small subunit ribosomal RNA gene, complete sequence	100
		JN222978.1	Acanthamoeba sp. 283 18S ribosomal RNA gene, partial sequence	99
		DQ087320.1	Acanthamoeba sp. S4 18S ribosomal RNA gene, partial sequence	99
2	A_i4	AY148954.1	Acanthamoeba sp. KA/E5 18S ribosomal RNA gene, complete sequence	99
		AY173014.1	Acanthamoeba sp. KA/MSS6 18S ribosomal RNA gene, complete sequence	99
		AY173013.1	Acanthamoeba sp. KA/MSS2 18S ribosomal RNA gene, complete sequence	99
		AY026243.1	Acanthamoeba polyphaga strain ATCC30461 small subunit ribosomal RNA gene, complete sequence	99
		JN222978.1	Acanthamoeba sp. 283 18S ribosomal RNA gene, partial sequence	98
		DQ087320.1	Acanthamoeba sp. S4 18S ribosomal RNA gene, partial sequence	98
3	E_i8 and C_i3	AY148954.1	Acanthamoeba sp. KA/E5 18S ribosomal RNA gene, complete sequence	98
		AY173014.1	Acanthamoeba sp. KA/MSS6 18S ribosomal RNA gene, complete sequence	98

		AY173013.1	Acanthamoeba sp. KA/MSS2 18S ribosomal RNA gene, complete sequence	98
		AY026243.1	Acanthamoeba polyphaga strain ATCC30461 small subunit ribosomal RNA gene, complete sequence	98
		JN222978.1	Acanthamoeba sp. 283 18S ribosomal RNA gene, partial sequence	98
		DQ087320.1	Acanthamoeba sp. S4 18S ribosomal RNA gene, partial sequence	98
4	E_i1, E_i4, F_i1, C_i2, D_i2, and CC2_Soil_s2	AY148954.1	Acanthamoeba sp. KA/E5 18S ribosomal RNA gene, complete sequence	97
		AY173014.1	Acanthamoeba sp. KA/MSS6 18S ribosomal RNA gene, complete sequence	97
		AY173013.1	Acanthamoeba sp. KA/MSS2 18S ribosomal RNA gene, complete sequence	97
		AY026243.1	Acanthamoeba polyphaga strain ATCC30461 small subunit ribosomal RNA gene, complete sequence	97
		JN222978.1	Acanthamoeba sp. 283 18S ribosomal RNA gene, partial sequence	97
		DQ087320.1	Acanthamoeba sp. S4 18S ribosomal RNA gene, partial sequence	97
5	A_i5	AY148954.1	Acanthamoeba sp. KA/E5 18S ribosomal RNA gene, complete sequence	96
		AY173014.1	Acanthamoeba sp. KA/MSS6 18S ribosomal RNA gene, complete sequence	96
		AY173013.1	Acanthamoeba sp. KA/MSS2 18S ribosomal RNA gene, complete sequence	96
		AY026243.1	Acanthamoeba polyphaga strain ATCC30461 small subunit ribosomal RNA gene, complete sequence	96
		JN222978.1	Acanthamoeba sp. 283 18S ribosomal RNA gene, partial sequence	96
		DQ087320.1	Acanthamoeba sp. S4 18S ribosomal RNA gene, partial sequence	96

6	F_i3, D_i1 and CC2_Soil_s1	AY148954.1	Acanthamoeba sp. KA/E5 18S ribosomal RNA gene, complete sequence	99
		AY173014.1	Acanthamoeba sp. KA/MSS6 18S ribosomal RNA gene, complete sequence	99
		AY173013.1	Acanthamoeba sp. KA/MSS2 18S ribosomal RNA gene, complete sequence	99
		AY026243.1	Acanthamoeba polyphaga strain ATCC30461 small subunit ribosomal RNA gene, complete sequence	99
		JN222978.1	Acanthamoeba sp. 283 18S ribosomal RNA gene, partial sequence	98
		DQ087320.1	Acanthamoeba sp. S4 18S ribosomal RNA gene, partial sequence	98
		U07407.1	Acanthamoeba polyphaga BCM:0173:16 ATCC 50371 18S rRNA gene	99
7	A_i6 and B_i2	AY148954.1	Acanthamoeba sp. KA/E5 18S ribosomal RNA gene, complete sequence	97
		AY173014.1	Acanthamoeba sp. KA/MSS6 18S ribosomal RNA gene, complete sequence	97
		AY173013.1	Acanthamoeba sp. KA/MSS2 18S ribosomal RNA gene, complete sequence	97
		AY026243.1	Acanthamoeba polyphaga strain ATCC30461 small subunit ribosomal RNA gene, complete sequence	97
		JN222978.1	Acanthamoeba sp. 283 18S ribosomal RNA gene, partial sequence	97
		DQ087320.1	Acanthamoeba sp. S4 18S ribosomal RNA gene, partial sequence	97
		U07407.1	Acanthamoeba polyphaga BCM:0173:16 ATCC 50371 18S rRNA gene	97
8	A_i7, E_i7, B_i1, HC1_Air_s4	AY148954.1	Acanthamoeba sp. KA/E5 18S ribosomal RNA gene, complete sequence	97
		AY173014.1	Acanthamoeba sp. KA/MSS6 18S ribosomal RNA gene, complete sequence	97
		AY173013.1	Acanthamoeba sp. KA/MSS2 18S ribosomal RNA gene, complete sequence	97

		AY026243.1	Acanthamoeba polyphaga strain ATCC30461 small subunit ribosomal RNA gene, complete sequence	97
		JN222978.1	Acanthamoeba sp. 283 18S ribosomal RNA gene, partial sequence	96
		DQ087320.1	Acanthamoeba sp. S4 18S ribosomal RNA gene, partial sequence	96
		U07407.1	Acanthamoeba polyphaga BCM:0173:16 ATCC 50371 18S rRNA gene	97
9	A_i8 and B_i4	GU596994.1	Acanthamoeba polyphaga isolate A8/SB2 18S ribosomal RNA gene, partial sequence	96
		AY148954.1	Acanthamoeba sp. KA/E5 18S ribosomal RNA gene, complete sequence	99
		AY173014.1	Acanthamoeba sp. KA/MSS6 18S ribosomal RNA gene, complete sequence	99
		AY173013.1	Acanthamoeba sp. KA/MSS2 18S ribosomal RNA gene, complete sequence	99
		AY026243.1	Acanthamoeba polyphaga strain ATCC30461 small subunit ribosomal RNA gene, complete sequence	99
10	HC2_Air_s6	GU596994.1	Acanthamoeba polyphaga isolate A8/SB2 18S ribosomal RNA gene, partial sequence	96
		AY148954.1	Acanthamoeba sp. KA/E5 18S ribosomal RNA gene, complete sequence	98
		AY173014.1	Acanthamoeba sp. KA/MSS6 18S ribosomal RNA gene, complete sequence	98
		AY173013.1	Acanthamoeba sp. KA/MSS2 18S ribosomal RNA gene, complete sequence	98
		AY026243.1	Acanthamoeba polyphaga strain ATCC30461 small subunit ribosomal RNA gene, complete sequence	98

11	E_i6 and B_i5	GU596994.1	Acanthamoeba polyphaga isolate A8/SB2 18S ribosomal RNA gene, partial sequence	96
		AY148954.1	Acanthamoeba sp. KA/E5 18S ribosomal RNA gene, complete sequence	96
		AY173014.1	Acanthamoeba sp. KA/MSS6 18S ribosomal RNA gene, complete sequence	96
		AY173013.1	Acanthamoeba sp. KA/MSS2 18S ribosomal RNA gene, complete sequence	96
		AY026243.1	Acanthamoeba polyphaga strain ATCC30461 small subunit ribosomal RNA gene, complete sequence	96
12	E_i2 and B_i3	GU596994.1	Acanthamoeba polyphaga isolate A8/SB2 18S ribosomal RNA gene, partial sequence	98
		JN222978.1	Acanthamoeba sp. 283 18S ribosomal RNA gene, partial sequence	98
		DQ087320.1	Acanthamoeba sp. S4 18S ribosomal RNA gene, partial sequence	98
		AY148954.1	Acanthamoeba sp. KA/E5 18S ribosomal RNA gene, complete sequence	98
		AY173014.1	Acanthamoeba sp. KA/MSS6 18S ribosomal RNA gene, complete sequence	98
		AY173013.1	Acanthamoeba sp. KA/MSS2 18S ribosomal RNA gene, complete sequence	98
		AY026243.1	Acanthamoeba polyphaga strain ATCC30461 small subunit ribosomal RNA gene, complete sequence	98
13	E_i5 and C_i1	AY148954.1	Acanthamoeba sp. KA/E5 18S ribosomal RNA gene, complete sequence	97
		AY173014.1	Acanthamoeba sp. KA/MSS6 18S ribosomal RNA gene, complete sequence	97
		AY173013.1	Acanthamoeba sp. KA/MSS2 18S ribosomal RNA gene, complete sequence	97

		AY026243.1	Acanthamoeba polyphaga strain ATCC30461 small subunit ribosomal RNA gene, complete sequence	97
		JN222978.1	Acanthamoeba sp. 283 18S ribosomal RNA gene, partial sequence	97
		GQ397478.1	Acanthamoeba sp. AcaVN16 18S ribosomal RNA gene, partial sequence	97
		EF140630.1	Acanthamoeba sp. KA/E29 18S ribosomal RNA gene, complete sequence	97
		DQ087320.1	Acanthamoeba sp. S4 18S ribosomal RNA gene, partial sequence	97
		DQ013363.1	Acanthamoeba polyphaga 18S ribosomal RNA gene, partial sequence	97
		AY173004.1	Acanthamoeba sp. KA/MSG4 18S ribosomal RNA gene, complete sequence	97
		U07407.1	Acanthamoeba polyphaga BCM:0173:16 ATCC 50371 18S rRNA gene	97
14	HC1_Soil_s2 and HC2_Soil_s3	GU596995.1	Acanthamoeba polyphaga isolate A10/SB2 18S ribosomal RNA gene, partial sequence	97
		AY148954.1	Acanthamoeba sp. KA/E5 18S ribosomal RNA gene, complete sequence	97
		AY173014.1	Acanthamoeba sp. KA/MSS6 18S ribosomal RNA gene, complete sequence	97
		AY173013.1	Acanthamoeba sp. KA/MSS2 18S ribosomal RNA gene, complete sequence	97
		AY026243.1	Acanthamoeba polyphaga strain ATCC30461 small subunit ribosomal RNA gene, complete sequence	
15	HC1_Soil_s5 and CC2_Soil_s4	U94741.1	Acanthamoeba lenticulata strain PD2S 18S small subunit ribosomal RNA gene, partial sequence	98
		U94740.1	Acanthamoeba lenticulata strain 25/1 18S small subunit ribosomal RNA gene, partial sequence	98
		U94738.1	Acanthamoeba lenticulata strain NJSP-3-2 18S small subunit ribosomal RNA	98

			gene, partial sequence	
		U94737.1	Acanthamoeba lenticulata strain 53-2 18S small subunit ribosomal RNA gene, partial sequence	98
		U94736.1	Acanthamoeba lenticulata strain 118 18S small subunit ribosomal RNA gene, partial sequence	98
		U94735.1	Acanthamoeba lenticulata strain E18-2 18S small subunit ribosomal RNA gene, partial sequence	98
		U94734.1	Acanthamoeba lenticulata strain 407-3a 18S small subunit ribosomal RNA gene, partial sequence	98
		U94733.1	Acanthamoeba lenticulata strain 68-2 18S small subunit ribosomal RNA gene, partial sequence	98
		AB525818.1	Acanthamoeba sp. AH-2009 gene for 18S ribosomal RNA, partial sequence, strain: NHE(Ac)1-IR-T5	98
		EU146072.1	Acanthamoeba sp. S35 18S ribosomal RNA gene, partial sequence	98
16	HC2_Soil_s4	U94741.1	Acanthamoeba lenticulata strain PD2S 18S small subunit ribosomal RNA gene, partial sequence	97
		U94740.1	Acanthamoeba lenticulata strain 25/1 18S small subunit ribosomal RNA gene, partial sequence	97
		U94738.1	Acanthamoeba lenticulata strain NJSP-3-2 18S small subunit ribosomal RNA gene, partial sequence	97
		U94737.1	Acanthamoeba lenticulata strain 53-2 18S small subunit ribosomal RNA gene, partial sequence	97
		U94736.1	Acanthamoeba lenticulata strain 118 18S small subunit ribosomal RNA gene, partial sequence	97

		U94735.1	Acanthamoeba lenticulata strain E18-2 18S small subunit ribosomal RNA gene, partial sequence	97
		U94734.1	Acanthamoeba lenticulata strain 407-3a 18S small subunit ribosomal RNA gene, partial sequence	97
		U94733.1	Acanthamoeba lenticulata strain 68-2 18S small subunit ribosomal RNA gene, partial sequence	97
		AB525818.1	Acanthamoeba sp. AH-2009 gene for 18S ribosomal RNA, partial sequence, strain: NHE(Ac)1-IR-T5	96
		EU146072.1	Acanthamoeba sp. S35 18S ribosomal RNA gene, partial sequence	96
17	CC1_Soil_s1, CC1_Soil_s5, HC2_Soil_s5, and CC2_Soil_s3	U94741.1	Acanthamoeba lenticulata strain PD2S 18S small subunit ribosomal RNA gene, partial sequence	96
		U94740.1	Acanthamoeba lenticulata strain 25/1 18S small subunit ribosomal RNA gene, partial sequence	96
		U94738.1	Acanthamoeba lenticulata strain NJSP-3-2 18S small subunit ribosomal RNA gene, partial sequence	96
		U94737.1	Acanthamoeba lenticulata strain 53-2 18S small subunit ribosomal RNA gene, partial sequence	96
		U94736.1	Acanthamoeba lenticulata strain 118 18S small subunit ribosomal RNA gene, partial sequence	96
		U94735.1	Acanthamoeba lenticulata strain E18-2 18S small subunit ribosomal RNA gene, partial sequence	96
		U94734.1	Acanthamoeba lenticulata strain 407-3a 18S small subunit ribosomal RNA gene, partial sequence	96
		U94733.1	Acanthamoeba lenticulata strain 68-2 18S small subunit ribosomal RNA gene,	96

			partial sequence	
		AB525818.1	Acanthamoeba sp. AH-2009 gene for 18S ribosomal RNA, partial sequence, strain: NHE(Ac)1-IR-T5	96
		EU146072.1	Acanthamoeba sp. S35 18S ribosomal RNA gene, partial sequence	96
18	HC2_Soil_s1 and	JQ031557.1	Acanthamoeba sp. AcaKM01 18S ribosomal RNA gene, partial sequence	97
	HC2_Soil_s6	GU936484.1	Acanthamoeba sp. M3 YH-2010 18S ribosomal RNA gene, partial sequence	97
		DQ992185.1	Acanthamoeba sp. EFW4 18S ribosomal RNA gene, partial sequence	97
		DQ992184.1	Acanthamoeba sp. EFW2 18S ribosomal RNA gene, partial sequence	97
		DQ992183.1	Acanthamoeba sp. EFW8 18S ribosomal RNA gene, partial sequence	97
		DQ992182.1	Acanthamoeba sp. EFW6 18S ribosomal RNA gene, partial sequence	97
		DQ103890.1	Acanthamoeba sp. MZOR 18S ribosomal RNA gene, partial sequence	97
		AY694141.1	Acanthamoeba sp. L749/98 18S ribosomal RNA gene, complete sequence	97
		AY148956.1	Acanthamoeba sp. KA/E8 18S ribosomal RNA gene, complete sequence	97
		AY026749.1	Acanthamoeba sp. isolate U/E10 small subunit ribosomal RNA gene, complete sequence	97
		AF019062.1	Acanthamoeba polyphaga Nagington 18S ribosomal RNA gene, partial sequence	97
19	HC2_Air_s3	AY026245.1	Acanthamoeba sp. U/H-C1 small subunit ribosomal RNA gene, complete sequence	99
		GQ380408.2	Acanthamoeba sp. cvX 18S ribosomal RNA gene, partial sequence	99

口試委員之意見回覆

陳建先老師	意見回覆
泳池採了 12 個樣本，採樣點的時間怎麼選擇?	Water samples were collected from six swimming pools during normal operation in Taipei, Taiwan (July 2 and July 16) of 2011 and (early spring March 21 and early spring March 28) of 2012. Sampling time of four community-type swimming pools were in the morning around 09:00 a.m. to 10:00 a.m. Sampling time of two school-type swimming pools were in the afternoon around 02:00 p.m. to 03:00 p.m.
泳池採樣時間點人數多少?各泳池間採樣時人數是否差不多?	The people density in four community-type swimming pools were around 0.048 to 0.112 (people/m^2).The people density in two school-type swimming pools were around 0.068 to 0.094 (people/m^2).
以環境衛生來說，就水樣而言這個環境的處理是水沒有處理好，還是由人貢獻的?	6.2.2.1. Pool water
論文中圖裡面只講到 cyst，是否真的有看到 trophozoite?移動性是根據 trophozoite 還是 cyst? 多久觀察一次?	4.3.5. Evaluation of the level of *Acanthamoeba* growth for thermotolerance and osmotolerance testing
引用文獻的方法須加以修正，方法學敘述，很少有人同一個方法後面引用一堆文獻，通常只會有一個。	Revised
文插圖的方式，整段寫完再看到圖，較好觀看閱讀。	Revised
文獻引用規則，按照字首或年代? 須統一。	Revised

演化樹有點複雜，不要讓演化樹那麼長，太長很難看，要扁平化一點，放太多反而失焦了。建議把 bootstrap value 小於 65 的先遮掉。	5.2.5. Phylogenetic analysis and genotyping
很長的表考慮放置附錄	See appendix
簡單總和一下，這三種樣本所得到的 Ac 到底有什麼地方是比較顯著不同的，分離株不同。	7.1. Conclusions

許晃慕老師	意見回覆
為什麼要把採樣器放正中央?	In order to represent each onion farmland
空氣採樣為什麼用這個採樣器?	Comparisons between biosampler IOM and biosampler MAS-100 on quantifications of *Acanthamoeba* by real-time PCR revealed that the Ct (cycle threshold) of sampling by IOM are more close to detection limit than MAS-100, results in the difficulty to determine *Acanthamoeba* concentrations. Therefore, MAS-100 was used as in the present study.
為何選擇洋蔥田?	Onion harvesters in the HengChun Peninsula of southern Taiwan simultaneously reported suffering from fungal corneal ulcers may attribute to ocular trauma caused by pieces of onion skin or plant leaves that drifted into their eyes while they were harvesting onions (Lin et al., 1999). Trauma has been known to the major risk factors especially in farmers with AK (Ma et al., 1981; Manikandan et al., 2004; Sun et al., 2006).
顯微鏡的圖都是 cyst 有點可惜，trophozoite 比較不會誤認，200x 倍率太小應該選擇更高的倍率	Photos in 1000x were taken.

選擇溫度的理由太弱，應可以參考其它的文獻。	At 30^{o}C (as control) (Caumo et al., 2009), 37^{o}C (Caumo et al., 2009). *Acanthamoeba* isolates can survive and grow in human bodies which have an average body temperature of ±37^{o}C, and are therefore considered as potential human pathogenic isolates. And 42^{o}C for 10 days (Chan et al., 2011) in order to investigate *Acanthamoeba* for increased thermotolerance. To investigate the effects of higher temperature on viability of *Acanthamoeba*, cysts were exposed 52^{o}C (Chan et al., 2011) for 24 hours prior to incubations at 30^{o}C for nine days, which favored excystation.
游泳池的 Ac 來源?	6.2.2.1. Pool water
游泳池的水質標準？可以與衛生局做對照。	6.2.7.1. Pool water

嵇達德老師	意見回覆
是否能追蹤 Ac 量的關係? 泳池已經是危險的那有多危險?	Future work
同地點空氣與土壤原蟲間的親緣關係異同?土壤中品種比較多有可能跟空氣採到的原蟲不一樣	Table 17 Alignment results of different isolates from the same sampling day and same sampling site
論文寫作問題，文獻更正，mannitol 應加上。	Revised
做一個縮寫表	See abbreviation
可跟恆春醫院結合知道農夫眼睛感染的狀況	Future work

張靜文老師	意見回覆

同日期同地點測的空氣土壤所分離的菌種做個比較，耐熱耐滲透壓的特性比較。

6.2.6. The potentially pathogenicity of *Acanthamoeba* isolated from the environments

結論必須要做條列式回應，加上建議未來可以努力的。

Chapter 7 Conclusions and Suggestions

Air 跟 soil 分離菌株之相關性，背景資料等等。

Table 17 Alignment results of different isolates from the same sampling day and same sampling site