Molecular, Immunological and Drug Sensitivity Studies of Pathogenic Free-living Amoebae

Thesis submitted for the degree of
Doctor of Philosophy
at the University of Leicester

by

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November 2011

Abstract

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Balamuthia mandrillaris is a free-living amoeba that causes *Balamuthia* amoebic encephalitis, a fatal disease in humans and animals. Its ecology and risk to humans is largely unknown, although *Balamuthia* infections have been mostly associated with soil-related activities. Ecology studies are hampered by difficulty in isolating the amoeba by culture methods used for other free-living amoebae. In this study, a DNA extraction method and nested PCR was developed for rapid detection of *B. mandrillaris* from environmental samples, without needing primary culturing. More than 25% of soil samples were positive for *B. mandrillaris*, predominantly those from high temperature countries. Additionally, *B. mandrillaris* was frequently found in thermally polluted water, with almost 50% of samples positive.

To facilitate the isolation of *B. mandrillaris* from environmental samples, immunomagnetic separation with *B. mandrillaris* antibodies was investigated. For this, poly- and monoclonal antibodies were produced and tested for specificity. Immunomagnetic separation isolated *B. mandrillaris* cysts but they were often contaminated with fungi, thus hindering further culture. In contrast, the technique was not suitable for the trophozoites due to toxicity and inability of the amoeba to survive the separation process.

Treatment of *B. mandrillaris* infections is mainly with combinations of drugs of varying efficacy and with undesirable side-effects. Here, an improved *in vitro* drug sensitivity assay was developed for *B. mandrillaris* trophozoites and cysts. Diminazene aceturate was shown to be effective against trophozoites and cysts, with the minimum amoebacidal concentration of 7.8 µM and minimum cysticidal concentration of 62.5 µM.

Naegleria fowleri is a thermophilic amoeboflagellate that causes primary amoebic meningoencephalitis, a fatal disease of the CNS. Conventional culture methods for its isolation from environmental samples may take several days, and thus a direct DNA extraction and rapid one-step nested PCR was utilised. With this, 15% of thermally polluted water samples were found positive for *N. fowleri*.

Acknowledgements

First and foremost, I would like to thank God for His eternal love, blessings in my daily life and given me the strength to complete my study. I am heartily thankful to my main supervisor, Dr. Simon Kilvington for his guidance, advices and ideas. My deepest gratitude goes to my co-supervisor, Prof. Peter Andrew who is also the Head of the Department of Infection, Immunity and Inflammation, University of Leicester for his invaluable discussions, motivation and supervision during the secondment of my main supervisor in California, USA.

The Ministry of Higher Education Malaysia and University of Malaya, Malaysia for funding my Ph.D study in the UK. The Head of the Parasitology Department, University of Malaya, Prof. Rohela Mahmud, for encouraging me to pursue my Ph.D study. Special thanks to Dr. Elizabeth Jane Shaw, Dr. Marco R. Oggioni and Dr. Maria Leonor Faleiro for providing soil samples from the UK (University of Reading farm), Italy and Portugal.

I would also like to thank to the staff of the Department of Infection, Immunity and Inflammation, especially Sheila Hewitt for arranging my meetings, Sushila Rambhai for processing my purchase orders and Malgorzata Goralik for her assistance in the Category 3 laboratory. Grateful thanks to Dr. Roger James and Dr. Mafalda Pires Damaso for their assistance and technical advice in the immunological part of this work. To my labmates (Lab G28) and friends: Dr. James Lonnen, Dr. Wayne Heaselgrave, Nale Yakubu, Victoria Attah, Jenny Geddes, Luke Andrew, Sandra Germano, Vitor Fernandes and MASLEICS, I would like to thank for their moral support and help.

Last but not least, I am greatly indebted and thankful to my beloved husband, Saypul Bahtiar Ruslan and my daughter, Nur Amierah Aleeya for their understanding, sacrifice, and encouragement throughout my PhD study. To my parents, siblings and my mother-in law, thanks for their unconditional love and continuous prayers.

List of Publications

Ahmad, A. F., Andrew, P. W., Kilvington, S., 2011. Development of a Nested PCR for Environmental Detection of the Pathogenic Free-Living Amoeba *Balamuthia mandrillaris*. *J Eukaryot Microbiol*, **58**, 269-271.

Ahmad, A. F., Lonnen, J., Andrew, P. W., Kilvington, S., 2011b. Development of a rapid DNA extraction method and one-step nested PCR for the detection of *Naegleria fowleri* from the environment. *Water Res*, **45**, 5211-5217.

List of Abbreviations

% - percent

°C - degrees centigrade

µg – microgram

A - Absorbance

AK – Acanthamoeba keratitis

ATCC – American Type Culture Collection

BAE –Balamuthia amoebic encephalitis

bp - base pair

BSA – bovine serum albumin

CCAP – Culture Collection of Algae and Protozoa

CDC – Centers for Disease Control and Prevention

CEP – California Encephalitis Project

cm - centimetre

CNS – central nervous system

CO_2 – carbon dioxide

CRB – Cambridge Research Biochemicals

CT - computed tomography

CTAB – hexadecyltrimethylammonium bromide

ddH_2O Double-distilled water

DMEM – Dulbecco's Modified Eagle Medium

DNA - deoxyribonucleic acid

dNTPs - deoxynucleosides

DPBS – Dulbecco A phosphate buffered saline

EA.hy926 – human umbilical vein cell line

ECCC – European Collection of Cell Cultures

EDTA - Ethylenediamine tetraacetic acid

ELISA – enzyme-linked immunosorbent assay

FBS – foetal bovine serum

fg -fentogram

FLA – free-living amoebae

g - gram

g - relative centrifugal force

GAE – Granulomatous amoebic encephalitis

HAT – hypoxanthine, aminopterin, thymidine

HBMEC – human brain microvascular endothelial cells

Hep-2 – human cervix carcinoma cell line

HGPRT – hypoxanthine-guanine phosphoribosyltransferase

hrs - hours

IIF – indirect immunofluorescent

ITS – internal transcribed spacers

kb - kilobase

kDA – kilo Dalton

LB - Luria broth

LSU – large subunit

M - Molar

MA104 – African green kidney monkey cell line

MCC – minimum cysticidal concentration

mg - microgram

min - minute

ml - millilitre

mm - millimetre

mM - millimolar

MRI – magnetic resonance imaging

MTAC – minimum trophozoite amoebacidal concentration

NaCl - sodium chloride

ng – nanogram

nm – nanometre

NNA- non-nutrient agar

No. – number

PAM – Primary amoebic meningoencephalitis

PCR – polymerase chain reaction

pg - picogram

PVPP – polyvinylpolypyrrolidone

rpm – revolutions per minute

rRNA – ribosomal ribonucleic acid

RT – room temperature

SDS - sodium dodecyl sulfate

sec - second

SSU – small subunit

TGS – Tris-glycine-SDS

UV – ultraviolet

v/v - volume by volume

w/v - weight by volume

µl - microlitre

µM micromolar

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1 Introduction

The study of free-living amoebae (FLA) stems from the development of early microscopes in the 17th century by Antony van Leeuwenhoek (1632-1723) who observed microorganisms, including protozoa for the first time (Porter, 1976, Khan, 2008). FLA are single-celled protozoa usually characterised as having two stages in their life-cycle, known as a feeding and replicating trophozoite and a dormant cyst (Page, 1988). However, in certain genera, the trophozoites are able to transform into a temporary stage, called flagellate, which is motile (Page, 1988). FLA are present almost ubiquitously in nature, including soil, water and air (John, 1993, Schuster *et al.*, 2004c). They feed by preying on bacteria, algae, fungi or other protists (Rodriguez-Zaragoza, 1994). Generally, most of them are not harmful to humans or animals but some have shown the ability to cause serious infections, which may even lead to death, as recently reviewed (Visvesvara *et al.*, 2007). Page (1974) used the term 'amphizoic' to show the ability of these FLA to exist as free-living and endoparasites. Among the representatives are members of the genera *Acanthamoeba*, *Balamuthia*, *Naegleria* and *Sappinia*. The classical taxonomy scheme for these free-living amoebae, which was mainly based on morphological characteristics, is shown in Figure 1.1A. Recently, a new scheme of taxonomy that is based on molecular phylogenetics has been proposed for protists, with a simpler hierarchical system (Adl *et al.*, 2005). The taxonomy for the four genera of FLA, under this new scheme, is shown in Figure 1.1B.

Acanthamoeba is the most ubiquitously present FLA, with the pathogenic species able to cause encephalitis, mostly in immunocompromised hosts (Marciano-Cabral *et al.*, 2003a). In addition, *Acanthamoeba* spp. are also responsible for Acanthamoeba keratitis, a painful sight-threatening infection in individuals with corneal trauma or

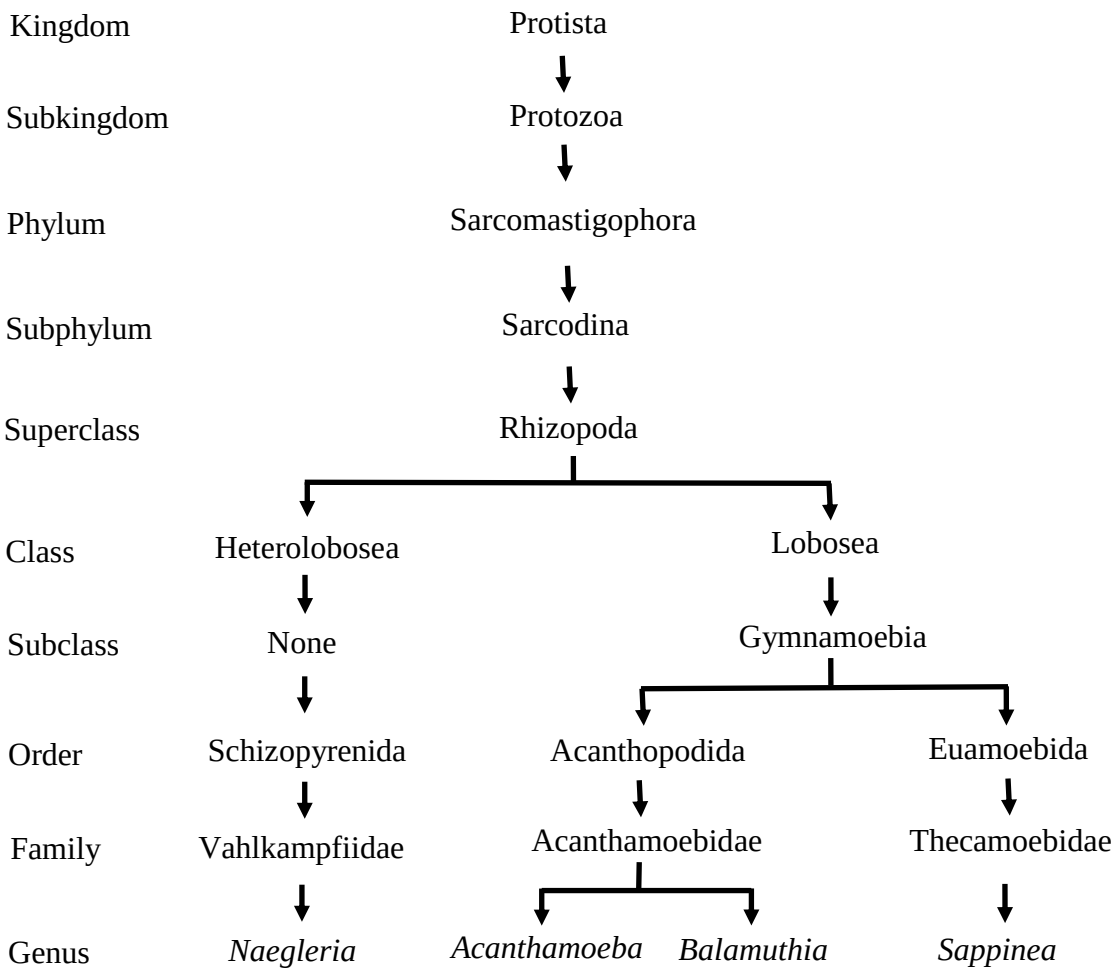
more commonly in contact-lens wearers with poor hygiene (Radford *et al.*, 2002). Pathogenic *Naegleria fowleri* causes a rapid and fatal meningoencephalitis in healthy individuals with a history of water-related activities, especially during summer months of the year (CDC, 2008b). *Balamuthia* is the third genus of FLA that is able to cause fatal encephalitis to human and animals (Visvesvara *et al.*, 2007). Unlike *Acanthamoeba* and *Naegleria*, *Balamuthia* causes infection in both immunocompromised and apparently healthy individuals (Matin *et al.*, 2008). Worryingly, the infection can occur throughout the year and is mostly associated with soil-related activities (Deetz *et al.*, 2003, CDC, 2008a). Recently, a fourth genus of FLA, *Sappinia* has also been associated with encephalitis in humans but only a single reported case in an immunocompetent male has been reported (Gelman *et al.*, 2001). This genus will not be discussed in this study.

1.1 *Balamuthia mandrillaris*

1.1.1 History, life-cycle and morphology

Balamuthia mandrillaris was first isolated in 1986 from the brain of a pregnant mandrill baboon that died at the San Diego Zoo Wild Animal Park due to meningoencephalitis (Visvesvara *et al.*, 1990a). The genus name was given as an attribution to the late Professor William Balamuth for his involvement in the studies of free-living amoebae. The species name shows the source of the first isolated strain (Visvesvara *et al.*, 1993). Initially, due to morphological resemblance to soil amoebae, the genus *Balamuthia* was placed under the family of Leptomyxidae (Visvesvara *et al.*, 1990a). However, analysis of rRNA sequences has shown that the amoeba has a close relationship with *Acanthamoeba* and therefore is now placed in the family Acanthamoebidae (Amaral Zettler *et al.*, 2000, Stothard *et al.*, 1998).

(A)



(B)

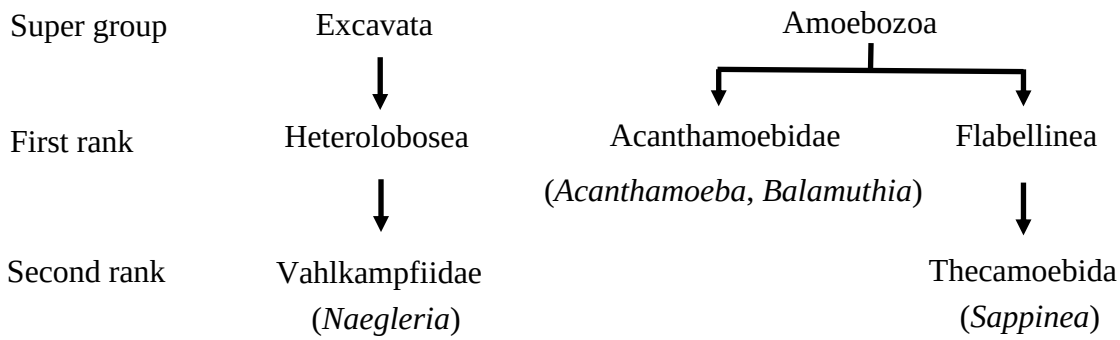


Figure 1.1 (A) Classical taxonomy scheme for free-living amoebae associated with disease in humans (Page, 1988, Ma *et al.*, 1990, Schuster *et al.*, 2004b, Matin *et al.*, 2008) and (B) New proposed taxonomy scheme for *Acanthamoeba*, *Balamuthia*, *Naegleria* and *Sappinea* (Adl *et al.*, 2005).

Currently, *B. mandrillaris* is the only known species belonging to the genus *Balamuthia* (Booton *et al.*, 2003a).

The life-cycle (Figure 1.2) consists of an infective, feeding trophozoite stage and a dormant cyst stage. The trophozoites are pleomorphic or have 'branching' morphology with sizes from 12 to 60 µm and they reproduce by binary fission (Matin *et al.*, 2008, Visvesvara *et al.*, 1993). Most of the trophozoites are uninucleate, although binucleates have been seen occasionally (Visvesvara *et al.*, 1993). Recently, the presence of multiple nuclei in *B. mandrillaris* pseudopodia has also been demonstrated (Dunnebacke, 2010). Two distinct patterns of locomotion are seen: either by formation of broad pseudopodia or spider-like movement using radiating pseudopods (Visvesvara *et al.*, 1993). The cysts, which lack pores, measure from 6 to 30 µm and have a triple-layered cell wall (Visvesvara *et al.*, 1993). By electron microscopy, the cell walls are characterised as a thin, wavy ectocyst (outer wall), a fibrous mesocyst (middle wall) and a thick endocyst (inner wall) (Visvesvara *et al.*, 1990a, Visvesvara *et al.*, 1993). The cysts are resistant to hostile conditions, such as extreme temperatures, ultra-violet (UV) radiation, disinfectants and antibiotics (Siddiqui *et al.*, 2008b).

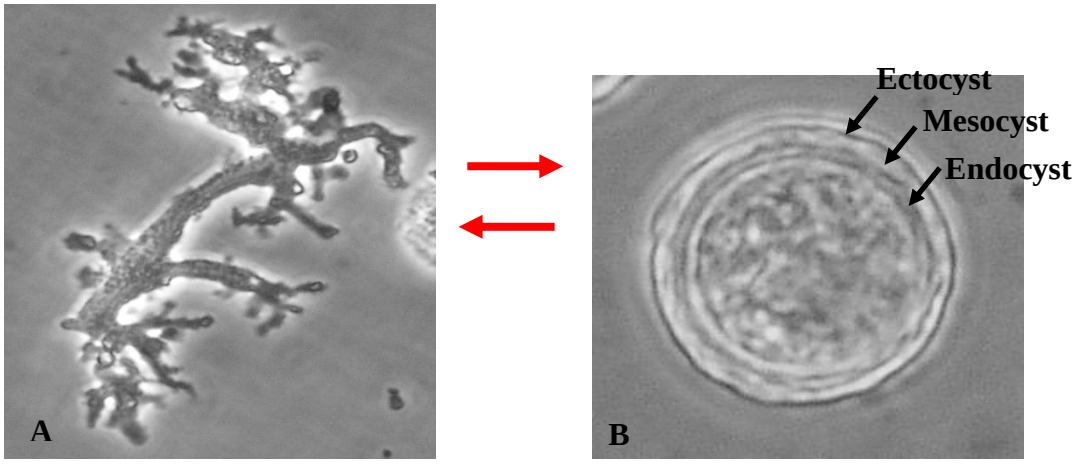


Figure 1.2 Life-cycle of *B. mandrillaris*. **A** Trophozoites (× 400); **B** Cyst (× 400)

1.1.2 Infection and disease

Balamuthia mandrillaris is the etiological agent for Balamuthia amoebic encephalitis (BAE), a fatal central nervous system (CNS) disease in both humans and animals. It is a chronic disease with an incubation period between a few weeks to a few years (Bravo *et al.*, 2006). The specific symptoms of BAE include headache, fever, skin lesions, lethargy, nausea, weight loss, walking difficulty, personality change, breathing difficulties, intracranial hypertension, cranial nerve palsy, hemiparesis, loss of consciousness and seizures (Siddiqui *et al.*, 2008a). Furthermore, the amoeba is also able to cause a serious cutaneous disease that may appear on the face, trunk or limbs (Bravo *et al.*, 2006, Gordon *et al.*, 1992). These painless skin lesions are characterised as rubbery to hard consistency plaques with size of few millimetres thick and one to several centimetres wide (Bravo *et al.*, 2006). The portals of entry to the host include breaks in the skin, nasal passages and inhalation of airborne cysts to the lung (Schuster *et al.*, 2008a). Eventually, amoebae may enter the bloodstream and haematogenously spread to the brain, causing death within weeks (Siddiqui *et al.*, 2008a). The amoebae may also bypass the circulatory system and enter the CNS directly via olfactory nerves (Kiderlen *et al.*, 2004). For example, a 64 year old male in California was suspected to have been infected through contaminated soil that entered the body through a prick caused by a rose thorn (Deetz *et al.*, 2003). In Thailand, a fatal human BAE case was associated with the entry of *B. mandrillaris* through a lesion on the nose after falling into a swamp during a motorbike accident (Intalapaporn *et al.*, 2004). Another mode of infection is via the oral route, as shown by the presence of *B. mandrillaris* antigen and trophozoites in brain sections of experimental mice (Kiderlen *et al.*, 2007). In Mississippi and Arizona, fatal infections have been reported through transplants of kidney, pancreas and liver (CDC, 2010a, CDC, 2010b).

Cases of balamuthiasis have also been reported in animals such as the gibbon, ape, orang-utan, gorilla, baboon, horse, sheep and dog (Anderson *et al.*, 1986, Canfield *et al.*, 1997, Finnin *et al.*, 2007, Foreman *et al.*, 2004, Fuentealba *et al.*, 1992, Kinde *et al.*, 1998, Rideout *et al.*, 1997, Visvesvara *et al.*, 1990a). Among the disease signs are sudden onset of depression, lethargy, lack of appetite, excess salivation and staggering, apparent head pain, whimpering, drowsiness, muscular weakness and coma (Anderson *et al.*, 1986, Visvesvara *et al.*, 1990a, Canfield *et al.*, 1997, Kinde *et al.*, 1998)

1.1.3 Ecology and epidemiology

Balamuthia mandrillaris is thought to be commonly present in soils and possibly water. To date, only three environmental strains have been isolated: two from Californian soils and one from a dust sample in Iran (Dunnebacke *et al.*, 2003, Dunnebacke *et al.*, 2004, Schuster *et al.*, 2003, Niyyati *et al.*, 2009). The soil strains (RP5 and OK1) of *Balamuthia* were isolated from an indoor flowerpot and an outdoor potted plant of a BAE victim in California, respectively. Potting soils are usually high in organic nutrients such as chicken fertilizer and vermicast, and these may provide a niche environment for bacterial growth and thus facilitate the growth of organisms that feed on them (Dunnebacke *et al.*, 2004). This would eventually increase the food supply (smaller free-living amoebae) for *Balamuthia* in the soil ecosystem. Attempts to isolate *B. mandrillaris* from water sources have been unsuccessful, although there have been two cases of BAE in dogs with previous history of swimming in pond water (Finnin *et al.*, 2007, Foreman *et al.*, 2004). In this study, the presence of *B. mandrillaris* in soils and water samples was explored using a nested PCR, using total DNA extracted directly from the environmental samples.

More than 150 cases of BAE have been reported worldwide, although the exact number might be higher due to unreported or undiagnosed cases. The BAE has been identified in Latin America (Peru, Chile, Argentina, Brazil, Mexico and Venezuela) (Cuevas *et al.*, 2006, Galarza *et al.*, 2002, Martinez *et al.*, 1994, Oddó *et al.*, 2006, Riestra-Castaneda *et al.*, 1997), United States (California, Texas, New York and Arizona) (Katz *et al.*, 2000, Bakardjiev *et al.*, 2003, Deol *et al.*, 2000, Duke *et al.*, 1997, Griesemer *et al.*, 1994, Healy, 2002), Asia (India, Japan, Thailand and Australia) (Intalapaporn *et al.*, 2004, Prasad *et al.*, 2008, Reed *et al.*, 1997, Shirabe *et al.*, 2002) and Europe (Czech Republic, Portugal and the United Kingdom) (Kodet *et al.*, 1998, Tavares *et al.*, 2006, White *et al.*, 2004). High temperature is thought as an important factor for its occurrence, with a high incidence of *Balamuthia* infections having been reported from Southern California and South America (CDC, 2008a, Seas *et al.*, 2006, Schuster *et al.*, 2006b).

Individuals at risk have been reported among the immunocompromised (HIV/AIDS, cancer or diabetes patients, alcoholics, intravenous drug users and those undergoing steroid treatment or organ transplantation) and immunocompetent (young children and older individuals) hosts and persons who are exposed to contaminated soil (Schuster *et al.*, 2008a). Moreover, individuals of Hispanic origin are more exposed to the disease since many of them are workers in the agriculture or construction sectors (Schuster *et al.*, 2004a, Schuster *et al.*, 2009). In a survey of 46 BAE cases, males (70%) were apparently more susceptible to the disease (Siddiqui *et al.*, 2008a). Air also might be a possible vehicle for transmission. Cases of BAE have been reported in young males in southern California, resulted from riding in an open car across desert terrain and riding on a motorcycle (Schuster *et al.*, 2006b).

1.1.4 Diagnosis

Due to the unfamiliar morphology of the amoeba and lack of expertise, most of BAE cases are diagnosed only at autopsy (Bakardjiev *et al.*, 2003, Jayasekera *et al.*, 2004). The lesions in the brain can be observed by brain imaging using magnetic resonance imaging (MRI) and computed tomography (CT) (Denney *et al.*, 1997, Healy, 2002, Deetz *et al.*, 2003). However, BAE and the cutaneous lesions can often be misdiagnosed as other diseases, including neurotuberculosis, neurocysticercosis, leishmaniasis, sporotrichosis, lupus vulgaris and Wegener's granulomatosis (Bravo *et al.*, 2006, Seas *et al.*, 2006). Miscroscopy is often used to detect the presence of *B. mandrillaris* in CSF, haematoxylin-eosin stained of brain or skin biopsies. Nevertheless, expertise is needed to differentiate *B. mandrillaris* from macrophages or other free-living amoebae (Guarner *et al.*, 2007).

At present, the gold standard for laboratory diagnosis of BAE is indirect immunofluorescent (IIF) staining of brain tissue sections using polyclonal antibodies raised in rabbits (Visvesvara *et al.*, 1990a, Deetz *et al.*, 2003). This technique has recently resolved a BAE case in a 22-year old, male dental student in New Delhi, India that initially could not be diagnosed upon autopsy (Prasad *et al.*, 2008). In addition, serum titres (concentrations) of *Balamuthia* antibodies in BAE patients can also be revealed using the IIF (Schuster *et al.*, 2006b). Other immunological methods used include enzyme-linked immunosorbent assay (ELISA). The assay was used with a group of 130 out of 431 samples of hospitalised encephalitis patients during the California Encephalitis Project (CEP) in 2006-2007. The CEP was introduced in 1998 to identify the etiological agents and verify the clinical and epidemiologic characteristics related to encephalitis (Glaser *et al.*, 2006). The ELISA results were

shown to be consistent to those obtained by IIF, although a few samples showed cross-reactivity due to unidentified antibodies in the serum (Schuster *et al.*, 2008b).

Recently, polymerase chain reaction (PCR) and sequencing have become favourite diagnostic tools for identifying *B. mandrillaris* from clinical and environmental samples (Booton *et al.*, 2003c, Foreman *et al.*, 2004). In a genotyping study by Booton *et al*., three human and one horse *B. mandrillaris* isolates from the United States were shown to have identical nuclear 18S rRNA gene sequences (Booton *et al.*, 2003a). However, analysis using mitochondrial 16S rRNA gene sequences from these four *B. mandrillaris* isolates and three additional human isolates (two from the United States and one from Australia) resulted in very low variation (0-1.8%) among the 7 isolates (Booton *et al.*, 2003a). This work eventually led to the development of mitochondrial 5' Balspec 16S and 3' Balspec 16S primers, which are specific for genus *Balamuthia* producing a PCR product of 1075 bp (Booton *et al.*, 2003c, Booton *et al.*, 2003b). Utilisation of this primer set with cerebrospinal fluid samples from BAE patients produced a band at the expected size of 1075 bp, however multiple bands of different sizes were also observed, possibly due to amplification of other DNA materials present in the samples (Yagi *et al.*, 2005). In addition, the primers set was not able to amplify DNA extracted from formalin-fixed brain and kidney tissue samples probably due to cross-linking and DNA fragmentation caused by the fixation process (Booton *et al.*, 2003c, Foreman *et al.*, 2004). This has prompted the identification of another primer (mt900) that is used together with the 5' Balspec 16S primer in a semi-nested PCR. Reamplification of the primary PCR product using 5' Balspec 16S/ 3' Balspec 16S primers with the semi-nested primers produced a smaller PCR product with size of 500 bp (Booton *et al.*, 2003c). In another study, Bal16Sr610 primer was designed and successfully used with the 5' Balspec 16S primer for amplifying DNA from formalin-fixed specimens yielding

a 230-bp PCR product (Foreman *et al.*, 2004). For *B. mandrillaris* isolated from environmental samples, PCR confirmation of the amoeba was commonly performed with mitochondrial 5' Balspec 16S and 3' Balspec 16S primers (Dunnebacke *et al.*, 2003, Schuster *et al.*, 2003, Niyyati *et al.*, 2009) . However, *B. mandrillaris* are usually adapted for growth on mammalian cells or axenic medium prior to PCR for removal of contaminants including bacteria, fungi and other amoebae (Dunnebacke *et al.*, 2003, Schuster *et al.*, 2003). This has limited the study of *B. mandrillaris* distribution in the environment because a long time is usually needed from initial isolation of the amoeba on non-nutrient agar plates lawned with *E. coli* before they can be transferred for growth on mammalian cells or in axenic medium (see Section 1.1.4.1). In addition, due to the large size of the PCR product (>1000 bp), more time is needed for the PCR reaction and thus it may not be suitable for use for rapid screening of large numbers of environmental samples (Booton *et al.*, 2003b, Booton *et al.*, 2003c). More recently, a triplex real-time TaqMan PCR was developed for simultaneous amplification of 18S rRNA gene of *Acanthamoeba* spp., *B. mandrillaris* and *N. fowleri* (Qvarnstrom *et al.*, 2006). The assay has been tested with DNA extracted from CSF and brain tissue of patients with neurological infections and results (positive for *N. fowleri*) were consistent with those obtained by immunofluorescence assay and culture (Qvarnstrom *et al.*, 2006). However, the amplification efficiency was reduced when used with unpurified DNA extracted from brain specimen containing high blood residues (Qvarnstrom *et al.*, 2006). Although fast results could be obtained in less than 5 hours using this rapid and sensitive real-time PCR assay technique (Qvarnstrom *et al.*, 2006), the machine and reagents are expensive and not suitable to be used for diagnosis of *Balamuthia* infection in developing countries. In addition, the suitability of the assay for environmental studies was not mentioned (Qvarnstrom *et al.*, 2006).

1.1.4.1 *In vitro* cultivation of *B. mandrillaris*

Unlike *Naegleria* and *Acanthanmoeba*, *Balamuthia* does not feed on either Gram-negative or Gram-positive bacteria. This limits the study of ecology of *B. mandrillaris* to be performed as the conventional cultivation technique for free-living amoebae involves the use of a non-nutrient agar with a lawn of *Escherichia coli* as the food source (Page, 1988, Schuster, 2002). Despite this, three environmental strains of *B. mandrillaris* have been isolated using this conventional technique but the process was tedious because it needed frequent observation using microscopy and *B. mandrillaris* could only be detected following a long incubation (weeks), possibly after feeding on other amoebae growing on the culture plates (Dunnebacke *et al.*, 2004). In addition, the presence of fungal contamination or other amoebae might make the detection of *B. mandrillaris* difficult and hence areas of agar containing *B. mandrillaris* need to be frequently transferred to fresh NNA-*E. coli* plates (Dunnebacke *et al.*, 2004).

In vitro studies have shown that *B. mandrillaris* feeds on mammalian cell monolayers, such as green monkey kidney cells and human brain microvascular endothelial cells (HBMEC) (Visvesvara *et al.*, 1993, Matin *et al.*, 2006a), rather than bacteria. However, the process of transferring *B. mandrillaris* isolated on NNA-*E. coli* plates onto mammalian cell monolayers is very challenging because bacteria present on the plate can contaminate the monolayer culture (Dunnebacke *et al.*, 2004). To overcome this, an intermediate step can be performed by transferring a small piece of agar containing *B. mandrillaris* onto another agar plates that have been coated with axenic *Naegleria* as the food source. The *Naegleria* used should not be able to survive at the temperature for culturing mammalian cells (Dunnebacke *et al.*, 2004). This procedure would eventually result with only *B. mandrillaris* growing on the monkey kidney cells (Dunnebacke *et al.*, 2004). In certain conditions, where other small amoebae were also seen growing on

the monkey kidney monolayer, further selection of *B. mandrillaris* needed to be done by dividing the culture in a 24-well plate. The plates were regularly inspected and wells containing only *B. mandrillaris* were transferred to fresh monkey kidney cell monolayers (Dunnebacke *et al.*, 2003). However, it may take time before the amoeba can be adapted for growth on monolayers. At first *B. mandrillaris* divides only slowly and can take up to 1 month to clear the mammalian cells, but thereafter they rapidly clear the monolayers on further transfers (Visvesvara *et al.*, 1993). This long process of adaptation may delay the undertaking of subsequent studies.

Besides *Naegleria*, *B. mandrillaris* is able to feed on *Acanthamoeba* but the ratio of *Acanthamoeba* to *Balamuthia* must be appropriate, otherwise higher numbers of *Acanthamoeba* may impede the growth of *Balamuthia* (Visvesvara *et al.*, 1993, Schuster *et al.*, 2004c). It was also reported that the growth of *B. mandrillaris* on *Acanthamoeba* monolayer was not as high as during culture on mammalian cells (Visvesvara *et al.*, 1993).

In 1996, an axenic (cell-free) medium called BM3 was developed by Schuster and Visvesvara for large scale growth of *Balamuthia*. In this the amoeba had a doubling time between 20 to 28 hours. The ingredients of this medium include Biosate peptone, yeast extract, Torula yeast ribonucleic acid (RNA), Hanks' balanced salts, liver digest, vitamins, lipid mixture, amino acids, glucose, hemin, taurine and newborn calf serum (Schuster *et al.*, 1996). In another study, a modified Chang's special medium, which was initially used for growing *Naegleria*, has been successfully used to culture *B. mandrillaris* (Kiderlen *et al.*, 2006). However, it has been reported that not all *B. mandrillaris* strains could be grown or maintained in an axenic culture medium (Dunnebacke *et al.*, 2004). In addition, prolonged cultivation in axenic medium has been reported to affect the cytopathogenicity of the amoebae (Kiderlen *et al.*, 2006).

1.1.4.2 Direct DNA extraction from environmental samples

Problems in studying microbial ecology due to uncultivable microorganisms have been well documented (Amann *et al.*, 1995). The emergence of molecular-based technique provides a new opportunity for detecting such microbes, provided an inhibitor-free DNA template is used. Therefore, direct DNA extraction of microorganisms without initial isolation from soils and sediments has been introduced (Ogram *et al.*, 1987, Holben, 1994). Over the past 30 years, various direct DNA extraction techniques have been developed for different types and compositions of environmental samples. Most authors have preferred combinations of physical disruption techniques (e.g. freeze-thawing, bead-beating or sonication) either with chemical lysis (e.g. ammonium acetate precipitation, hexadecyltrimethylammonium bromide, detergents and salts) or enzyme treatments (e.g. proteinase K or lysozyme) (Carrigg *et al.*, 2007, Courtois *et al.*, 2001, Fortin *et al.*, 2004, Miller *et al.*, 1999, Ogram *et al.*, 1987, Schneegurt *et al.*, 2003, Steffan *et al.*, 1988). However, problems have been reported due to co-extraction of contaminants, particularly humic acids that may interfere with PCR amplification (Zhou *et al.*, 1996). To overcome this, further purification steps utilising polyvinylpolypyrrolidone (PVPP) (Ogram, 1998, Holben, 1994, Lakay *et al.*, 2007), hexadecyltrimethylammonium bromide (CTAB) (Zhou *et al.*, 1996) or caesium chloride density gradients (Holben *et al.*, 1988) have been reported. In microbial ecology studies, DNA dilution prior to PCR was not recommended because it leads to loss of species diversity, yielding DNA amplification mostly from those microorganisms that lysed easily during DNA extraction (Stach *et al.*, 2001). The use of silica-magnetite nanoparticles and commercial kits has also recently been applied as an alternative tool for extracting DNA from environmental samples (Roose-Amsaleg *et al.*, 2001, Sebastianelli *et al.*, 2008). The authors have reported that very low DNA yields, which

were hardly visible on agarose gel, were obtained from three soil samples, using a SoilMaster DNA extraction kit. In contrast, separation using silica-magnetic nanoparticles showed high DNA yields (Sebastianelli *et al.*, 2008). In another study, intact DNA fragments of high molecular weight DNA were obtained from a rhizosphere soil sample following DNA extraction using a Mo Bio soil kit. However, the kit was limited to use with small amounts of soils (0.25 g) (Maarit Niemi *et al.*, 2001).

1.1.5 Antimicrobial therapy of BAE and *Balamuthia* skin lesions and prevention of the diseases

Despite of more than 150 cases of *Balamuthia* infection having been reported, including those causing *Balamuthia* amoebic encephalitis (BAE) and skin lesions with or without the CNS involvement, only a few successful treatments have been reported (Schuster *et al.*, 2004d, Martinez *et al.*, 2010). Usually a combination of drugs was used. The drugs are thought to produce synergistic effects *in vivo* that may not be apparent during individual *in vitro* testing. However, not all infected individuals can be treated using the same drug regimen and early diagnosis is important to avoid fatal outcomes (Bakardjiev *et al.*, 2003, Deetz *et al.*, 2003, Seas *et al.*, 2006, Schuster *et al.*, 2004d) . To date, 12 balamuthiasis survivors have been reported: five from the United States, six from Peru and one from Australia (Deetz *et al.*, 2003, Jung *et al.*, 2004, Seas *et al.*, 2006, CDC, 2008a, Cary *et al.*, 2010, Martinez *et al.*, 2010, Botterill *et al.*, 2011). Four of the BAE patients in the United States survived after treatment with a combination of flucytosine (5-fluorocytosine), fluconazole, pentamidine isethionate, sulfadiazine, macrolide antibiotics (azithromycin or clarithromycin) and phenothiazines (thioridazine or trifluoperazine) (Deetz *et al.*, 2003, Jung *et al.*, 2004, Cary *et al.*, 2010). This combination of drugs was chosen based on the *in vitro* efficacy of the drugs when tested on clinical strains of *Balamuthia* (Schuster *et al.*, 1996, Schuster *et al.*, 2008a).

However, the use of some drugs, including trifluoperazine, thioridazine, pentamidine and fluconazole, was discontinued in some treatments due to side-effects. Prominent side effects are muscle rigidity, hyponatremia, hyperglycemia/increased creatinine and increased in hepatic enzymes levels (Deetz *et al.*, 2003, Cary *et al.*, 2010). In addition the use of azithromycin was changed to clarithromycin in two of the USA patients to increase the penetration of the drug to the CNS or to overcome the side effect of high creatinine level (Deetz *et al.*, 2003). The details of drug regimen for another survivor from the United States was not available (CDC, 2008a).

In Peru, two of the patients with skin and CNS involvement survived following prolonged drugs regimen including albendazole and intraconazole, with one patient also having a surgical resection of a large lesion on the chest to reduce the amoeba load (Bravo *et al.*, 2006, Martinez *et al.*, 2010). The details of treatments for the other two Peruvian survivors with CNS involvement were not reported (Seas *et al.*, 2006). In a rare *Balamuthia* infection case, a Peruvian patient who suffered from skin lesion on the face but without CNS involvement fully recovered without receiving any treatment, (Bravo *et al.*, 2006, Schuster *et al.*, 2008a). Recently, a female Peruvian survivor with skin lesions on both knees and BAE was successfully treated with a drug regimen including miltefosine, fluconazole and albendazole (Martinez *et al.*, 2010). In Australia, a patient with skin lesion and BAE recovered following treatment with itraconazole, flucytosine, sulfadiazine, liposomal amphotericin and azithromycin and had an open resection for removal of a brain abscess (Botterill *et al.*, 2011). The used of pentamidine in the patient's treatment was discontinued due to a side-effect of hypotension (Botterill *et al.*, 2011). Therefore, there is an urgent need to find new drugs that are effective in killing the amoeba without producing serious side-effects.

Eradication of *B. mandrillaris* is virtually impossible since they exist in the environment. This has been proved by the presence of *Balamuthia* antibodies in healthy human populations (Huang *et al.*, 1999, Kiderlen *et al.*, 2010). As most of the BAE patients have been actively involved in agriculture or construction work, wearing protective clothes or gloves is recommended (Schuster *et al.*, 2008a).

1.1.5.1 *In vitro* drug assay

A number of drugs have been tested against *B. mandrillaris* trophozoites *in vitro* and assays have been performed either in cell-free culture medium or on mammalian cell monolayers (Schuster *et al.*, 1996, Schuster *et al.*, 2006a). For drug assays using cell-free medium, trophozoites are incubated with known concentrations of drugs in a high nutrient growth medium. Samples of the medium would be removed at 1-3 days intervals to determine the *B. mandrillaris* counts. Subsequently the drug-treated trophozoites are inoculated on cells monolayer and the amoebastatic or amoebacidal activity of the drug confirmed by the ability of the drug-treated trophozoites to cause clearing of cell monolayers (Schuster *et al.*, 2006a). Alternatively, the drug-treated trophozoites can also be inoculated into fresh drug-free growth medium to determine their viability (Schuster *et al.*, 2006a).

The second type of assay involves incubation of *B. mandrillaris* trophozoites with different concentrations of drug while on the cell monolayers. After a few days, the drug-containing medium is removed and replaced with fresh culture medium. The culture plates are then checked for clearing of monolayers in order to determine the amoebastatic or amoebacidal activity of the drug (Schuster *et al.*, 1996).

Based on *in vitro* drug assays performed in a cell-free culture medium, pentamidine isethionate, propamidine isethionate, polymyxin B and gramicidin S at a concentration

of 10 µg/ml showed the highest percentage of inhibition (93 – 97%) of amoeba growth (Schuster *et al.*, 1996). However, amoebae treated with pentamidine isethionate and polymyxin B showed recovery following transfer to fresh medium and hence the drugs were concluded as amoebastatic and not amoebacidal. The two other drugs although have been confirmed as amoebacidal, are not recommended for parenteral use (Schuster *et al.*, 1996, Schuster *et al.*, 2008a). Recently, miltefosine has been reported to be inhibitory to amoeba growth at a minimum concentration of 65 µM and amoebacidal at a minimum concentration of 75 µM (Schuster *et al.*, 2006a). In contrast, another study has reported that miltefosine concentrations of up to 100 µM were only inhibitory to the trophozoites (Schuster *et al.*, 2008a). Variations in efficacy of drugs tested *in vitro* can be due to difference *B. mandrillaris* strains used (Schuster *et al.*, 2006a).

At present, there is only one report of activity against *B. mandrillaris* cysts (Siddiqui *et al.*, 2008b). The assay was only tested with 5 µg/ml and 10 µg/ml of pentamidine isethionate but there were no cysticidal effects, based on the ability of the cysts to transform into the trophozoite stage following inoculation on cell monolayers (Siddiqui *et al.*, 2008b).

1.2 *Naegleria*

1.2.1 History, life-cycle and morphology

The amoeba was first discovered in 1899 by Schardinger and was called *Amoeba gruberi*. The genus name *Naegleria* was later proposed in the early 20th century by Alexeieff (Alexeieff, 1912, Khan, 2008). At present, more than 30 species are identified in this genus, based on sequence analysis of small subunit ribosomal deoxyribonucleic acid (SSU rDNA), large subunit ribosomal DNA (LSU rDNA) and the internal transcribed spacer (ITS) regions, including the 5.8S rDNA (De Jonckheere, 2002, De

Jonckheere, 2004). However, only one species, *Naegleria fowleri*, has been linked with a rapidly fatal infection of the central nervous system (CNS) in humans, termed as primary amoebic meningoencephalitis (PAM) (see Section 1.2.2). *Naegleria fowleri* was named by Carter in 1970 as an acknowledgment to Doctor Malcolm Fowler who first recognised the disease in Australia (Carter, 1970). Previously, *N. fowleri* was also known as *N. aerobia*, mainly in India (Singh *et al.*, 1970), or *N. invades* (De Jonckheere, 2002). Two other pathogenic species, *N. australiensis* and *N. italica*, only killed mice and so far no human infection has been reported (De Jonckheere, 1981, De Jonckheere *et al.*, 1984).

As an amoeboflagellate, *Naegleria* has a flagellate stage in its life-cycle, along with the trophozoite and cyst stages (Figure 1.3). The transient flagellate stage is characterised as a pear-shaped cell with typically two flagella (Carter, 1970). The size of the trophozoites is between 10 to 20 µm and they are characterised by a prominent contractile vacuole in the cytoplasm for controlling the water content of the cell, and a nucleus with a large central nucleolus or karyosome (Carter, 1970, Ma *et al.*, 1990). The trophozoites of the pathogenic *N. fowleri* exhibit sucker-like structures, called amebostomes, which function to engulf food and for attachment to surfaces (Marciano-Cabral *et al.*, 1983, John *et al.*, 1985, John *et al.*, 1984). In contrast, non-pathogenic species of *Naegleria* have modified amebostome-like structures which are not as clearly defined as those from *N. fowleri* (John *et al.*, 1985). The movement of *Naegleria* trophozoites is rapid, with bluntly eruptive pseudopodia, and reproduction is by binary fission (John, 1993).

The flagellate stage has been described as a non-feeding or non-dividing stage (Marciano-Cabral, 1988). The flagellates play roles in distribution of amoebae in the environment and can be induced when the trophozoites are suspended in non-nutrient

medium, such as in distilled water or buffer (John, 1993, Preston *et al.*, 2003). However, it has been reported that some of the *Naegleria* species, including *N. chilensis, N. indonesiensis* and some *N. fowleri* strains, have either lost the ability to flagellate or the flagellates are capable of undergoing division (De Jonckheere *et al.*, 2001, De Jonckheere, 2002).

Cysts of *N. fowleri* are double-walled with pores, spherical in shape with sizes between 7-10 µm and they are often found clumped closely together (Carter, 1970, John, 1993). The cysts have been reported to be susceptible to desiccation, chlorination, pH changes, extremes temperatures and metabolic waste accumulation (Chang, 1978, Schuster, 2002). A concentration of 0.5 µg/ml of free chlorine was able to destroy the *N. fowleri* cysts after 1 hour of contact suggesting chlorination as an efficient method in controlling the occurrence of *N. fowleri* in swimming pools or drinking water (De Jonckheere *et al.*, 1976)

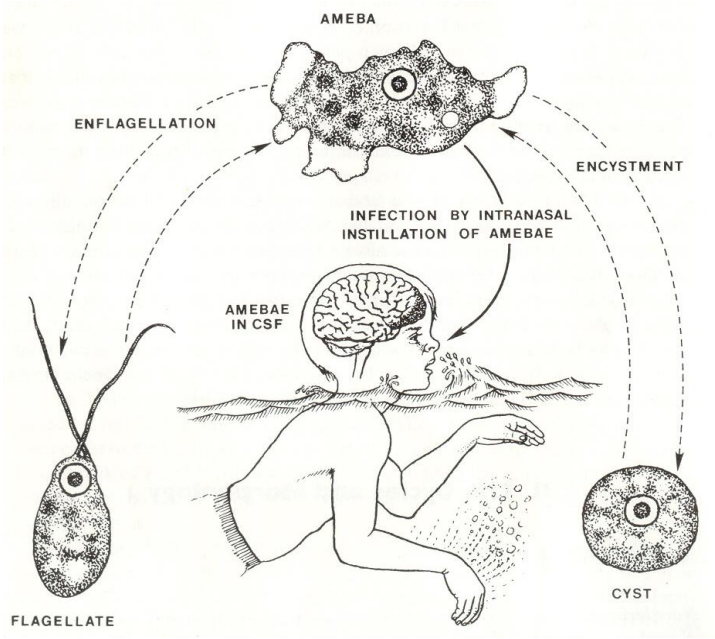


Figure 1.3 Three-stage life-cycle of *N. fowleri* and human infection (John, 1993)

1.2.2 Infection and disease

Naegleria fowleri causes an acute and fulminating CNS disease called primary amoebic meningoencephalitis (PAM) which results in death within 3-7 days following infection (John, 1982, Cain *et al.*, 1981). Infection can be acquired through inhalation of the pathogenic trophozoites, flagellates or cysts through the nasal passages. In the body, the cysts and flagellates revert to the trophozoite stage (John, 1993). The amoeba penetrates the olfactory neuroepithelium within the nasal cavity and migrates along the olfactory nerves and eventually invades the brain, causing hemorrhagic necrosis and oedema (Carter, 1970). The estimated number of human PAM cases from the 1960s until 2000 is approximately 200 (Schuster *et al.*, 2004b). The victims were usually healthy children and young adults with a recent history in water-related activities, such as swimming, diving or bathing (Lares-Villa *et al.*, 1993, Heggie, 2010). In the USA, out of 111 PAM cases occurred between 1962-2008, 79.3% (88 patients) were males aged between 10 and 14 years (Yoder *et al.*, 2010). Males are believed to be the majority because of their frequent involvement with water-related activities, compared to females (Yoder *et al.*, 2010). In Nigeria and Pakistan, infection has been reported to be acquired from sniffing water into the nasal passages during ritualistic washings before Muslim prayers (Lawande *et al.*, 1980, Shakoor *et al.*, 2011). PAM had also been reported due to inhalation of particles containing cysts of pathogenic *N. fowleri* during the dust ladden winds of the Harmattan period in Nigeria (Lawande *et al.*, 1979b).

It has been suggested that global warming might cause elevation of water temperatures and create suitable environments for *N. fowleri* (Cogo *et al.*, 2004). This would eventually increase the chances of being infected with the amoeba (Cogo *et al.*, 2004).

1.2.3 Ecology and epidemiology

Naegleria fowleri has been isolated from natural or man-made lakes, rivers, wells, hot springs, thermally polluted effluents of power plants, sewage, inadequately chlorinated swimming pool and even from nasal passages and throats of healthy individuals (Lawande *et al.*, 1979a, Kilvington *et al.*, 1995a, Kilvington *et al.*, 1997, Reveiller *et al.*, 2003, Blair *et al.*, 2008). As a thermophilic amoeba, *N. fowleri* can survive temperatures up to 45°C and is commonly present in artificial thermal habitats, rather than natural warm environments where non-pathogenic thermophilic *Naegleria* would be in abundance and create competition (De Jonckheere, 2002, Kilvington *et al.*, 1997). Nevertheless, the presence of other thermophilic amoebae in the same body of water may act as an indicator for the presence of *N. fowleri* (Lares-Villa *et al.*, 2010). *N. fowleri* also prefers areas with stagnant water and undisturbed by recreational activities (Jamerson *et al.*, 2009). Movement of water due to current or human activities may cause the trophozoites to transform into its dormant cyst stage and sink at the bottom of the water (Jamerson *et al.*, 2009). In the environment, the growth of *N. fowleri* is believed to be facilitated by iron and the presence of microbial iron chelators can affect their proliferation (Newsome *et al.*, 1983). In another study, *N. fowleri* numbers have been shown to be almost 60 times higher in cooling towers that are equipped with stainless steel condensers compared to those equipped with brass condensers, due to the release of copper into the water which has inhibitory effect on the amoeba growth (Pernin *et al.*, 2001).

PAM has been reported in many countries, including Australia, United Kingdom, Mexico, Italy, India, New Zealand, Thailand, Japan, Venezuela, Cuba, Madagascar, Africa, United States, Belgium, Brazil, Czechoslovakia, Hungary, New Guinea, Nigeria, Panama, Uganda, (Visvesvara *et al.*, 1990b, Cogo *et al.*, 2004, Wiwanitkit, 2004, Sugita

et al., 1999, Lares-Villa *et al.*, 1993). In the UK, a fatal PAM case was reported in 1978, in Bath, due to swimming in an indoor pool that was supplied with naturally warm spring water (Cain *et al.*, 1981). Recently, in Florida, Texas and Arizona, six fatal PAM cases have been reported in the summer months of 2007, presumably reflecting the fact that more people would be involved in recreational activities (CDC, 2008b, Yoder *et al.*, 2010).

1.2.4 Diagnosis

Microscopy remains the basic diagnostic tool for detecting the presence of *N. fowleri* trophozoites in clinical samples (Martinez *et al.*, 1997), although the presence of lesions in the brain of patients suspected of having PAM can be determined using computed tomography and magnetic resonance imaging (MRI) (Mackowiak *et al.*, 2010, Vargas-Zepeda *et al.*, 2005). Because *N. fowleri* feeds on bacteria, they can be isolated in the laboratory from both clinical and environmental samples by culturing the specimens on NNA-*E. coli* plates (Schuster, 2002). For environmental samples, incubation of water or sediment samples on NNA-*E. coli* plates at 44 °C is used to selectively grow *N. fowleri* from other non-thermophilic amoebae (Anon, 1990, Page, 1988). However, identification of the pathogenic *N. fowleri* based on selective incubation alone is not reliable because there have been reports of other thermophilic *Naegleria*, such as *N. lovaniensis* (De Jonckheere, 2002). *N. lovaniensis* is a non-pathogenic species but resembles *N. fowleri* in its ability to tolerate temperatures up to 45 °C, in causing cytopathic effects on tissue culture and antigenic relatedness (Stevens *et al.*, 1980, Marciano-Cabral, 1988, De Jonckheere, 2002).

An enflagellation test has commonly been used for genus confirmation of *Naegleria* spp. (Page, 1988). Nevertheless, there have been reports of several strains or species of

Naegleria spp. which are unable to enflagellate (De Jonckheere *et al.*, 2001, Behets *et al.*, 2003). In addition the method does not enable differentiation between pathogenic and non-pathogenic species of *Naegleria* (De Jonckheere, 2002). A mouse pathogenicity test has been used to differentiate *N. fowleri* from other species but the method is time-consuming and lacks specificity because *N. australiensis* and *N. italica* were also able to kill mice (De Jonckheere, 1981, De Jonckheere, 2002).

Molecular methods involving conventional and real-time PCRs have been used to differentiate pathogenic and non-pathogenic *Naegleria* species (Reveiller *et al.*, 2002, Pelandakis *et al.*, 2002, Robinson *et al.*, 2006, Qvarnstrom *et al.*, 2006). Conventional PCR assays with a sensitivity limit equivalent to a single cell of *N. fowleri* have been reported (Kilvington *et al.*, 1995a, Sparagano, 1993). However, both PCR assays required primary culturing and subculturing of amoebae on NNA-*E. coli* plates to remove PCR inhibitors (Kilvington *et al.*, 1995a, Sparagano, 1993). This is a time consuming process and lacks specificity because *N. fowleri* can be overgrown by more rapidly growing species, such as *N. lovaniensis*, and also other free-living amoebae (Kilvington *et al.*, 1995b, De Jonckheere, 2002, Maclean *et al.*, 2004). In addition, the PCR by Sparagano is laborious because the PCR products needed to be further confirmed with Southern blot hybridisation (Sparagano, 1993).

A nested PCR with a detection limit of 5 pg of *N. fowleri* DNA or 5 intact trophozoites has been developed. This has the advantage that the assay can be applied without the need of genomic DNA extraction (Reveiller *et al.*, 2002, Maclean *et al.*, 2004). However, the suitability of the nested PCR for use with water samples containing sediments was not mentioned (Reveiller *et al.*, 2002). In addition the nested PCR involves two separate PCR reactions which can be laborious, especially when working with high numbers of samples. It also is prone to cross-contamination during transfer of

primary PCR products to secondary PCR tubes (Reveiller *et al.*, 2002, Marciano-Cabral *et al.*, 2003b). A multiplex real-time PCR also has been described for simultaneous detection of *N. fowleri*, *Acanthamoeba* spp. and *B. mandrillaris* 18S rDNA, however the expensive chemicals and equipment required may limit its diagnostic use in developing countries (Qvarnstrom *et al.*, 2006). Although the 18S rDNA PCR has commonly been used in phylogenetic studies, sequence and riboprinting analyses showed very little differences between the *N. fowleri* and the non-pathogenic, thermophilic *N. lovaniensis* (De Jonckheere, 1994a, De Jonckheere, 1994b). This has led to the use of ribosomal internal transcribed spacers (ITS), which have less involvement in the ribosomal function than the rDNA genes thus allowing them to evolve more rapidly (Gerbi, 1985). This rapid evolution allows sequence polymorphism within the ITS region and therefore more suitable to be used for detecting nucleotide differences between and within *Naegleria* species (De Jonckheere, 1998, Pelandakis *et al.*, 2000). Again, primary culturing was a pre-requisite prior to PCR amplification (Pelandakis *et al.*, 2002). Recently, a real-time PCR has been reported that allows detection of *N. fowleri* to be performed with pipeline biofilms and drinking water samples, using total DNA extracted with a commercial soil kit without primary culture of the amoeba (Puzon *et al.*, 2009). Nevertheless, the suitability of the DNA extraction for use with water samples with high turbidity due to sediment and algae contamination needed further investigation (Puzon *et al.*, 2009).

1.2.5 Treatment and prevention

Amphotericin B appears to be the most preferable drug for treating PAM (Seidel *et al.*, 1982). The drug acts by causing abnormalities to the nuclear shape and mitochondria, increasing proliferation of both the rough and smooth endoplasmic reticula, decreasing the number of food vacuoles and enhancing the formation of autophagic vacuoles in the

amoebae (Schuster *et al.*, 1975). In addition, the drug has been shown to inhibit pseudopodia formation and induce blebbing of the *Naegleria* plasma membrane (Schuster *et al.*, 1975). The drug acts synergistically with miconazole (Seidel *et al.*, 1982). Nevertheless, the success of treatment has been reported to be dependent on other factors, including early diagnosis, prompt intervention and timing of initiation of antiamoebic therapy coupled with intensive supportive care (Seidel *et al.*, 1982).

In addition to adequate therapies, strategies to prevent infection also can be employed to control PAMs. Adequate and continuous chlorination of swimming pools and water supplies may prevent *N. fowleri* infections. For example, continuous free residual chlorine of 0.5 mg/litre of water has been proved to decrease the *N. fowleri* problem in public water supplies in South Australia (Dorsch *et al*., 1983). In addition, water-related activities, such as swimming in warm freshwater, should be avoided or undertaken with caution, such as using a nose plug or not immersing the head into the water, especially during summer months (Visvesvara *et al.*, 2007, Heggie, 2010).

1.3 *Acanthamoeba*

1.3.1 History and classification

In 1930, an amoeba was isolated from cultures of the yeast *Cryptococcus pararoseus* (Castellani, 1930). Based on morphology characterisation, the amoeba was at first placed in the genus *Hartmanella* and called *Hartmanella castellanii*. The genus name *Acanthamoeba* was later established, in 1931(Volkonsky, 1931). Since then, no reports of the amoeba were published until in the late 1950s, when *Acanthamoeba* was discovered as a tissue culture contaminant and associated for the first time to causing fatal infection in monkeys and mice (Culbertson *et al.*, 1958, Culbertson *et al.*, 1959, Jahnes *et al.*, 1957).

Initially, the genus was subdivided into three mains groups, based on size and the morphological characteristics of the cysts (Pussard *et al.*, 1977). Group I species (e.g. *A. astronyxis*, *A. comandoni* and *A. tubiashi*) are characterised by having the largest cysts of the genus, with an average diameter of ≥ 18 µm. Group II species (e.g. *A. castellanii*, *A. polyphaga*, *A. triangularis*, *A. griffini* and *A. hatchetti*) have cysts with a mean diameter of < 18 µm and the endocyst may be stellate, polygonal, triangular or sometimes round or oval in shape. Group III species (*A. palestinensis*, *A. culbertsoni*, and *A. royreba*) also have cysts with a mean diameter of < 18 µm but the ectocyst is either gently rippled or unrippled, with usually a round endocyst (Pussard *et al.*, 1977, Khan, 2008). Subsequently, it has been found that species identification by morphology alone is unreliable (Stratford *et al.*, 1978). Cyst morphology has been reported to change with environmental conditions, for example with change in ionic strength (Sawyer, 1971, Stratford *et al.*, 1978). In addition, the classification has limited value in describing pathogenicity because different strains from the same species can be virulent, weakly virulent or avirulent (Khan, 2008). Species identification employing immunological, biochemical and physiological criteria have also been used (Walochnik *et al.*, 2001, De Jonckheere, 1983). However, as many of the *Acanthamoeba* spp. show similar antigenic determinants and can change depending on culture conditions, results have been unconvincing for species identification (Weekers *et al.*, 1997, Walochnik *et al.*, 2001). The breakthrough of molecular techniques, particularly PCR and sequencing, has enabled a reliable classification of the genus *Acanthamoeba* to be done based by analysis of rDNA sequences. To date, 17 different genotypes (T1-T17) have been assigned in the genus (Stothard *et al.*, 1998, Corsaro *et al.*, 2010, Nuprasert *et al.*, 2010).

1.3.2 Life-cycle and morphological structures

As with other free-living amoebae, *Acanthamoeba* reproduce by binary fission and may exist as a trophozoite or cyst (Figure 1.4). The trophozoite is between 24-56 µm and is recognisable by the presence of spine-like projections from the cell surface, termed acanthopodia (Gr. *acanth* spine or thorn) (John, 1993, Page, 1988). The acanthopodia have been reported to have important roles in adherence and in the pathogenesis of *Acanthamoeba* infections (Khan, 2001, Khan, 2003). The trophozoite is commonly uninucleate with a large central nucleolus and it exhibits prominent contractile vacuole in the cytoplasm, which functions to control the water content of the cell (Marciano-Cabral *et al.*, 2003a). Locomotion is through formation of a hyaline pseudopodium and the movement is sluggish in all *Acanthamoeba* species (Preston *et al.*, 1984).

The dormant cyst morphology varies between the three *Acanthamoeba* groups. It is composed of a double-wall made up of the ectocyst and endocyst. It has a diameter of 12-25 µm (Page, 1988). The cyst is resistant to harsh conditions, such as radiation, antimicrobials, disinfectants and extreme temperatures (Aksozek *et al.*, 2002, Dart *et al.*, 2009, Coulon *et al.*, 2010). For example, *Acanthamoeba* cysts have been shown to retain viability after more than 20 years storage in water at 4 °C or desiccation at room temperature (Mazur *et al.*, 1995, Sriram *et al.*, 2008).

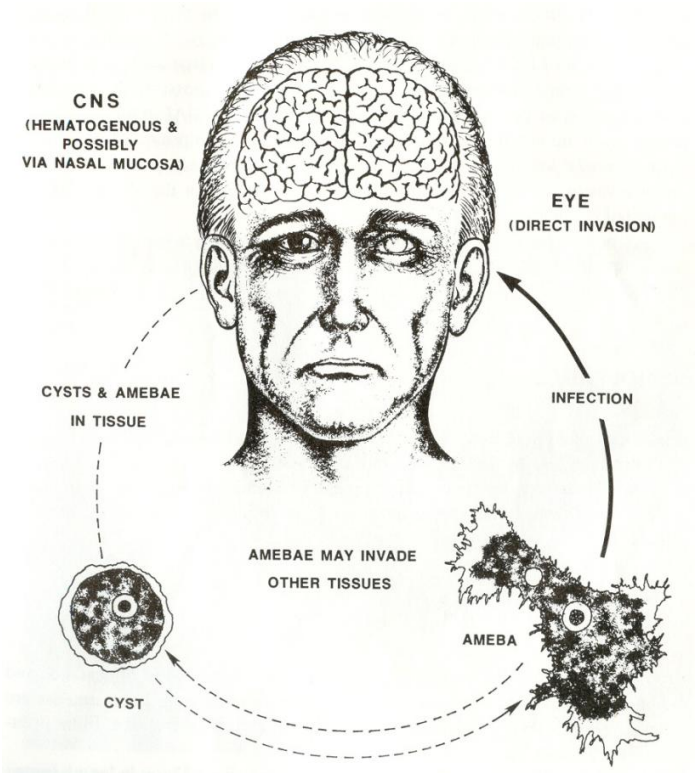


Figure 1.4 Life cycle and human infection of *Acanthamoeba* spp. (John, 1993)

1.3.3 Infections and diseases

Not all *Acanthamoeba* species are able to cause disease. The most common *Acanthamoeba* species that are associated with disease in humans are *A. castellanii*, *A. polyphaga*, *A. hatchetti*, *A. healyi* and *A. culbertsoni* (Bloch *et al.*, 2005, Sheng *et al.*, 2009, Walochnik *et al.*, 2000, Moura *et al.*, 1992). Two important diseases caused by these pathogenic amoebae are granulomatous amebic encephalitis (GAE) and Acanthamoeba keratitis (AK). Most of the GAE victims are immunodeficient and suffering from AIDS or conditions such alcoholism, chemotherapy, organ transplantation or bone marrow failure that predisposes them to opportunistic infections (Martinez, 1991). The disease can result from hematogenous spread following inhalation of the amoeba or skin lesions or directly through olfactory neuroepithelium (Khan, 2008).

Acanthamoeba keratitis is a painful inflammation of the eye and characterised as severe pain of the eye, photophobia, stromal ring infiltrate which if untreated can result in blindness (Illingworth *et al.*, 1998). The disease occurs mainly in contact lens wearers with poor hygiene or in developing countries, such as India, due to corneal trauma (Sharma *et al.*, 2000, Dart *et al.*, 2009, Ibrahim *et al.*, 2007).

Disseminated *Acanthamoeba* infections such as cutaneous lesions, sinusitis and otitis can also occur, depending on the immune status of the host (Oliva *et al.*, 1999, Marciano-Cabral *et al.*, 2003a).

1.3.4 Ecology

Acanthamoeba has been described as the most ubiquitous amoeba present in soil, water and air (Rodriguez-Zaragoza, 1994). The amoeba has been isolated from bottled water, dental treatment units, seawater, sewage, sand, beaches, marine sediments and vegetables (Lorenzo-Morales *et al.*, 2005, Munson *et al.*, 2006, Khan, 2008). In a cumulative study using 214 soil and dust samples collected in 11 countries, 93% were positive for *Acanthamoeba* (Mergeryan, 1991).

In the UK, Acanthamoeba keratitis has been associated with use of domestic tap water that is contaminated with *Acanthamoeba* (Kilvington *et al.*, 2004). Pathogenic *Acanthamoeba* spp. have also been isolated from dust collected from air conditioners in Malaysia (Chan *et al.*, 2011). Compared to other FLA, *Acanthamoeba* is able to survive high osmolarity and it has been isolated from Atlantic Ocean (Sawyer, 1980).

1.3.5 Detection methods

The ability of *Acanthamoeba* to feed on bacteria has enabled clinical and environmental strains to be isolated using NNA-*E. coli* plates (Page, 1988, Moura *et al.*, 1992). Microscopy is the basic tool used for identification of the amoeba at the genus level

based on the morphological characteristics shown by the trophozoites and cysts (Khan, 2008, da Rocha-Azevedo *et al.*, 2009). Subsequently, further confirmation is commonly performed by polymerase chain reaction (PCR) due to its sensitivity and reliable results (Booton *et al.*, 2005, Lorenzo-Morales *et al.*, 2005, Lehmann *et al.*, 1998). An 18S ribosomal RNA gene primer set (JDP1-JDP2) that is specific for the *Acanthamoeba* genus has been developed and is able to detect all known species of the genus (Schroeder *et al.*, 2001). PCR of mitochondrial DNA has also been used for detection of *Acanthamoeba* in clinical and environmental samples (Kilvington *et al.*, 2004, Yagi *et al.*, 2007). Recently, a real-time PCR assay has been developed for simultaneous detection of *Acanthamoeba* spp., *N. fowleri* and *B. mandrillaris* (Qvarnstrom *et al.*, 2006). However, sequence analysis is commonly required following most PCR assays for species identification and determining their association with other existing genotypes that are able to cause infection in humans (Schroeder *et al.*, 2001, Kilvington *et al.*, 2004).

1.4 Free-living amoebae as reservoirs for pathogenic bacteria

The relationship between FLA and bacteria is unique. Besides being the common predators in controlling bacterial populations in the soil, FLA also act as reservoirs for many pathogenic microorganisms (Rodriguez-Zaragoza, 1994, Greub *et al.*, 2004, Shadrach *et al.*, 2005, Walochnik *et al.*, 2005). Being inside FLA gives several advantages to the internalised microorganisms, often called endosymbionts. For example, increase in resistance to drugs and disinfectants or increased virulence in animal models (e.g. *Mycobacterium avium* grown in *A. castellanii* showed higher replication in the spleen and liver of infected mice compared to those infected with *M. avium* grown in 7H9 broth) (Cirillo *et al.*, 1997) and protection from harsh conditions once the amoebae encyst and facilitation of distribution in the environment (Cirillo *et*

al., 1997, Greub *et al.*, 2004, Khan, 2008). Among the well-studied endosymbionts is *Legionella pneuomophila*, which is the causative agent of Legionnaires' disease (Rowbotham, 1980). It has been suggested that an amoeba containing *Legionella* could be an infective particle for humans rather than the free form of *Legionella* (Rowbotham, 1980). In addition each vesicle in an amoeba could contain up to hundreds of *L. pneumophila* (Berk *et al.*, 1998). In other study, polyhexamethylene biguanide killed 99.99% of *L. pneuomophila* grown in broth cultures but only 90% were killed within 6 hours for those grown in *Acanthamoeba* (Barker *et al.*, 1992).

1.5 Polyclonal and monoclonal antibodies

Polyclonal antibodies are derived from multiple B cells and can be produced by immunising animals several times with the immunogen of interest. Subsequently, serum containing the polyclonal antibodies is prepared from the animal blood (Coligan *et al.*, 2005, Benjamini *et al.*, 2000). In contrast, monoclonal antibodies are derived from a single B cell. The technique for producing monoclonal antibodies, was pioneered by Georges Köhler and Cesar Milstein (Köhler *et al.*, 1975). The advantages of monoclonal antibodies over polyclonal antibodies are their mono-specificity, homogeneity and consistency (Lipman *et al.*, 2005). Classically, it involves fusion of B cells from the spleen of an immunised animal (typically a mouse) with myeloma cells, using polyethylene glycol. The combination of these two cells generates an immortal product (a hybridoma) with each one specific for a single epitope (Benjamini *et al.*, 2000). Selection of hybridoma following the fusion step is done using HAT medium containing hypoxanthine, aminopterin and thymidine. This medium is selective for hybridomas because of the lack of enzyme HGPRT (Hypoxanthine-guanine phosphoribosyltransferase) in myeloma cells (Løvborg, 1982). Because of this enzyme defect the myeloma cells cannot synthesis DNA via the salvage pathway and need to

use the *de novo* pathway which requires folic acid. Aminopterin in the HAT medium is a structural analogue of folic acid and inhibits folic acid dependent enzymes (Løvborg, 1982). Only hybridomas will survive in the HAT medium because B cells bring HGPRT positivity and thus the ability to use the salvage pathway for DNA synthesis, while myeloma cells bring immortality to the hybrids. Unfused B cells have a short lifespan due to apoptosis and unfused myeloma cells die due to inability to synthesis DNA (Løvborg, 1982).

Polyclonal antibodies against *Acanthamoeba* spp., *N. fowleri*, non-pathogenic *Naegleria* spp. and *B. mandrillaris* have been developed, mostly using whole or lysed amoebae or a mixture of trophozoites and cysts (Muldrow *et al.*, 1982, Flores *et al.*, 1990, Visvesvara *et al.*, 1993, Powell *et al.*, 1994, John *et al.*, 1998). Polyclonal antibodies have been regularly used in immunofluorescence assays, especially for confirmation of the presence of pathogenic *B. mandrillaris*, *Acanthamoeba* spp. and *N. fowleri* in clinical specimens, including brain and skin biopsies and cerebrospinal fluid (Flores *et al.*, 1990, Visvesvara *et al.*, 1993, Cogo *et al.*, 2004, da Rocha-Azevedo *et al.*, 2009, Schuster *et al.*, 2009). However, the use of polyclonal antibodies has been associated with lack of specificity due to cross-reactivity problems (Flores *et al.*, 1990, Guarner *et al.*, 2007). For example, Flores and colleagues reported a cross-reactivity of a *N. fowleri* polyclonal antibody with brain sections of a patient that had been confirmed to have granulomatous amoebic encephalitis caused by *Acanthamoeba* (Flores *et al.*, 1990). In another study using immunohistochemistry assay, a polyclonal antibody to *Acanthamoeba* was shown to cross-react with a brain biopsy of a patient diagnosed with Balamuthia amoebic encephalitis (Guarner *et al.*, 2007).

Polyclonal antibodies can also be used in indirect immunofluorescence assays for species identification of *Naegleria* species. John and his group have able to identify the

species of nineteen *Naegleria* isolates that were showed to be pathogenic in mice, using polyclonal antibodies developed against pathogenic and non-pathogenic *Naegleria* spp. Their findings showed that twelve isolates were identified as *N. australiensis*, six as *N. fowleri* and one as *N. lovaniensis*. However, the six *N. fowleri* isolates also showed some cross-reactivity with *N. lovaniensis* polyclonal antibodies, suggesting antigenic relatedness between the two species (John *et al.*, 1998). In another study, confirmation of two environmental isolates of *Balamuthia* was done using *Balamuthia* polyclonal antibody in an immunofluorescence assay (Schuster *et al.*, 2003, Dunnebacke *et al.*, 2003).

Polyclonal antibodies have been used in Western blotting assays for determining specific epitopes of pathogenic and non-pathogenic free-living amoebae including *A. culbertsoni*, *A. astronyxis*, *A. polyphaga*, *N. fowleri*, *Hartmanella vermiformis* and *Vahlkampfia avara* (Powell *et al.*, 1994). It was found in the study that protein bands with sizes of less than 18.5 kDa were only specific for *Acanthamoeba* species but minor cross-reactivity at different protein sizes were also seen between the three *Acanthamoeba* species tested. While *N. fowleri*, *H. vermiformis* and *V. avara* showed specific multiple protein bands between 18.5 - 106 kDa, 49.5 - 80 kDa and 32.5 - 106 kDa respectively, with no cross-reactivity (Powell *et al.*, 1994).

Monoclonal antibodies against *N. fowleri* and pathogenic *Acanthamoeba* spp. have been successfully produced (Visvesvara *et al.*, 1987, Sparagano *et al.*, 1993, Hiwatashia *et al.*, 1997, Flores *et al.*, 1990, Reveiller *et al.*, 2000). Among the common methods used to determine the specificity of monoclonal antibodies against both types of free-living amoebae are immunofluorescence assay, ELISA, Western blotting, radioimmunoprecipitation assay and flow-cytometry (Sparagano *et al.*, 1993, Reveiller *et al.*, 2000, Turner *et al.*, 2005). Choosing the appropriate method for specificity

testing is important because variation in results has been reported (Visvesvara *et al.*, 1987).

Two monoclonal antibodies (3A4 and 5D12), developed by Sparagano and colleagues, were very specific for *N. fowleri* and both showed reactivity against trophozoite, cyst and flagellate stages and not to those of non-pathogenic *Naegleria* spp. (Sparagano *et al.*, 1993). In another study, each of four monoclonal antibodies to *N. fowleri* (N3B, N4A, N5A and N6A) and *A. polyphaga* (AC2B, A3B, AC7B and AC9B) have been shown to be specific against the respective free-living amoeba and no cross-reactivity between the two genera of free-living amoebae were detected. However, two of the *A. polyphaga* monoclonal antibodies (AC7B and AC9B) also showed cross-reactivity to *A. castellanii* (Flores *et al.*, 1990), thus limiting their use for species identification. Cross-reactivity of monoclonal antibodies between *Acanthamoeba* species has also been reported by Turner *et al*. (2005), suggesting similarity of epitopes among the species. In that study, monoclonal antibodies against *A. castellanii* (Group II) reacted not only to cysts of another *A. castellanii* strain and *A. polyphaga* belonging to Group II species but also to cysts of *A. lenticulata* and *A. culbertsoni* of Group III species by flow-cytometry (Turner *et al.*, 2005).

As with polyclonal antibodies, both *Acanthamoeba* and *N. fowleri* monoclonal antibodies have been successfully used for confirmation of free-living amoebae in brain or cornea sections (Visvesvara *et al.*, 1987, Flores *et al.*, 1990, Reveiller *et al.*, 2003, Turner *et al.*, 2005). In addition, monoclonal antibodies have been used for specific identification of *N. fowleri* from environmental isolates either with immunofluorescence assay or ELISA (Reveiller *et al.*, 2003, Leiva *et al.*, 2008). To date, no monoclonal antibodies against *B. mandrillaris* have been reported.

1.6 Immunomagnetic separation of cells

The immunomagnetic separation method has successfully been applied in microbiology for isolation of bacteria such as *Escherichia coli* O157, *Listeria monocytogenes*, *Shigella dysenteriae, Vibrio parahaemolyticus* and *Salmonella* sp. either from stool or food samples (Parham *et al.*, 2003, Cudjoe *et al.*, 1997, Hibi *et al.*, 2006, Fluit *et al.*, 1995, Islam *et al.*, 1992, Tomoyasu, 1992). In parasitology, the method is commonly used for isolation of *Cryptosporidium parvum* and *Giardia lambia* cysts from biosolid and water samples (Massanet-Nicolau, 2003, Bukhari *et al.*, 1998). The detection and enumeration of *N. fowleri* from water samples has been reported using a combination of immunomagnetic separation and a solid-phase cytometer (Pourima *et al.*, 2002). In this study, the immunomagnetic separation method will be tested for their suitability in the isolation of trophozoite and cyst stages of *B. mandrillaris*.

1.7 Aims of study

Relatively little is known about the optimal culture conditions for *B. mandrillaris* and this has limited the study of ecology of the amoeba. Also current diagnostic tests and treatments have significant limitations, and so improvements are needed. In addition, an improved detection method for *N. fowleri* is also needed for rapid screening for the amoeba to replace the current detection method which relies on primary culturing prior to PCR. The aims of this study were therefore:

- To develop a direct DNA extraction method and *B. mandrillaris* nested PCR for rapid detection of the amoeba from environmental samples
- To test the presence of *N. fowleri* in water samples using the developed DNA extraction method and a one-step *N. fowleri* nested PCR

- To develop poly- and monoclonal antibodies against *B. mandrillaris* for use in immunomagnetic separation of the amoeba
- To develop a simple and reliable drug assay for *B. mandrillaris* trophozoites and cysts

2 Materials and methods

2.1 Chemicals

All chemicals used in this study were obtained from Sigma-Aldrich Company Ltd. (Dorset, UK) and Invitrogen Ltd. (Paisley, UK) unless otherwise stated. Sterilisation of solutions was performed either by autoclaving at 121°C for 15 min or passing through a 0.2 µm PALL filter membrane (VWR International Ltd., Lutterworth, UK).

2.2 Microorganisms, cell lines and plasmids

The details of all microorganisms, cell lines and plasmids used in this study are listed in Table 2.1.

Table 2.1 Microorganisms, cell lines and plasmid used in this study

Cell lines	**Strain/Source**
African green monkey kidney	MA104, ECCC 85102918
Human cervix carcinoma	Hep-2, ECCC 86030501
Human umbilical vein	EA.hy926, Dr. Robert Hirst, Leicester Royal Infirmary, UK
Amoebae	**Strain/Source**
Acanthamoeba castellanii	ATCC 50370
Acanthamoeba castellanii	ATCC 30234
Acanthamoeba polyphaga	ATCC 30461
Acanthamoeba polyphaga	Ros[#]
Acanthamoeba polyphaga	CCAP 1501/3G
Acanthamoeba hatchetti	CDC V573
Acanthamoeba sp.	SCSK09-01[#]
Acanthamoeba sp.	SCSK09-02[#]
Acanthamoeba sp.	SCSK09-03[#]
Acanthamoeba sp.	SCSK09-04[#]

Continued:

Amoebae	**Strain/Source**
Acanthamoeba sp.	SCSK09-05[#]
Acanthamoeba sp.	SCSK09-06[#]
Acanthamoeba sp.	SCSK09-07[#]
Acanthamoeba sp.	SCSK09-08[#]
Acanthamoeba sp.	SCSK09-09[#]
Balamuthia mandrillaris	CDC:V039, ATCC 50209
Naegleria lovaniensis	C0490[#]
Naegleria lovaniensis	SK 41/44/1[#]
Naegleria australiensis	AB-T-F3[#]
Naegleria gruberi	NEG-M, ATCC 30224
Naegleria fowleri	CCAP 1518
Naegleria fowleri	KUL, ATCC 30808
Naegleria fowleri	NH1[#]
Naegleria fowleri	MSM[#]
Bacteria	**Strain/ Source**
Escherichia coli	JM101[#]
Escherichia coli	JM109[#]
Staphylococcus aureus	ATCC 6538
Stenotrophomonas maltophilia	ATCC 15099
Bacillus sp.	JPN[#]
Fungus	**Strain/Source**
Candida albicans	ATCC 10231
Plasmid	**Strain/Source**
Naegleria fowleri	pUC PB2.3[#]

ECCC = European Collection of Cell Cultures (Salisbury, UK)

ATCC = American Type Culture Collection (LGC Standards, Teddington, UK)

CDC = Centers for Disease Control and Prevention (Atlanta, USA)

CCAP = Culture Collection of Algae and Protozoa (Scotland, UK)

[#] = In-house collection

2.3 Culture of *Acanthamoeba* spp.

All *Acanthamoeba* spp., except strains started with 'SCSK', were grown in a semi-defined axenic culture medium in Nunc tissue culture flasks (Fisher Scientific, Loughborough, UK) at 32°C. Culture medium was prepared as 1 litre volumes containing 20 g Biosate peptone (BD Biosciences, Oxford, UK) 5 g glucose, 0.3 g potassium dihydrogen orthophosphate (KH_2PO_4), 100 µl Vitamin B_{12} (100 µg/ml, Appendix 1), 3 ml L-Methionine (5 mg/ml, Appendix 1) and nanopure water. If necessary, pH was adjusted to 6.5-6.6 with 1M sodium hydroxide (NaOH, Appendix 1). The solution was aliquoted in 250 ml volumes in sterile Duran bottles and autoclaved. Prior to use, sterile-filtered penicillin/streptomycin was added to a final concentration of 100 U/ml and 0.1 mg/ml respectively. The complete medium was stored at 4°C and used within one month. The 'SCSK' strains were cultured on NNA-*E. coli* plates (Section 2.5) and incubated at 32°C.

2.4 *Escherichia coli* suspension

Escherichia coli strain JM101 was cultured on Tryptone Soy Agar (Appendix 1) and incubated at 37°C overnight. A single colony was picked using a sterile disposable loop and put into 100 ml of LB broth (Appendix 1) in a 175 cm^2 Nunc tissue culture flask (Fisher Scientific, Loughborough, UK). The flask was then incubated upright at 30°C in an orbital shaking incubator at 100 rpm (Sanyo Gallenkamp, Loughborough, UK) overnight. The culture was then poured evenly into two 50 ml polypropylene centrifuge tubes and centrifuged at 3000 × *g* for 30 min. The supernatant was discarded and pellet was resuspended in 30 ml of ¼ strength Ringer's solution (Appendix 1) by vortexing. The suspension was centrifuged for another 30 min at the same speed and supernatant

was discarded. The pellet was then suspended in 10 ml of ¼ strength Ringer's solution by vortexing and stored at 4°C for use within 14 days.

2.5 NNA-*E. coli* plates

Non-nutrient agar plates (NNA, Appendix 1) were inoculated with 3 drops of *E. coli* suspension (Section 2.4) using a sterile 3 ml pipette. Immediately, the suspension was spread evenly over the whole surface of the NNA using a sterile disposable hockey stick shaped spreader (Fisher Scientific, Loughborough, UK). The NNA-*E. coli* plates were allowed to dry at RT and stored at 4°C for use within 7 days.

2.6 Culture and manipulation of *N. fowleri*

All experiments including culturing, DNA extraction and cryopreservation of *N. fowleri* strains were performed in the Category 3 labs of the Department of Infection, Immunity and Inflammation, University of Leicester, UK. The growth medium used was modified SCGYEM (De Jonckheere, 1977) and flasks were incubated at 32°C. Two main solutions were initially prepared. For solution 1, the components were 10 g casein digest, 5 g yeast extract, 2.5 g glucose, 1.325 g sodium phosphate dibasic (Na_2HPO_4), 0.8 g potassium phosphate monobasic (KH_2PO_4) and nanopure water to 890 ml. If necessary the pH was adjusted to 6.9-7.0 and autoclaved. Solution 2 was prepared by adding 10 g neutralised liver digest (Oxoid Ltd., Hampshire, UK) and nanopure water to 100 ml. The pH was adjusted to pH 6.9-7.0 and filter sterilised. To obtain the complete medium, 890 ml of solution 1, 10 ml of solution 2, 100 ml of heat-inactivated foetal bovine serum (Fisher Scientific, Loughborough, UK) and 5 ml of penicillin/streptomycin (10, 000 U/ml/ 10, 000 mg/ml) was mixed. The complete medium was stored at 4°C.

2.7 Culture of others *Naegleria* spp.

Naegleria lovaniensis, *N. australiensis* and *N. gruberi* strains were cultured at 37°C, 30°C and 25°C respectively in modified PYNFH medium. Briefly, basal medium containing Biosate peptone (10 g), yeast extract (10 g), yeast nucleic acid (1 g), folic acid (15 mg), hemin (1 mg) and nanopure water (880 ml) was prepared and autoclaved. Next, buffer solution was prepared by adding 18.1 g potassium phosphate monobasic (KH_2PO_4) and 25 g sodium phosphate dibasic (Na_2HPO_4) to a volume of 1L of nanopure water and this was filter sterilised. Following this, 880 ml of the basal medium was mixed with 20 ml of buffer solution and kept at 4°C until used. Prior to use, 10% (v/v) of heat-inactivated foetal bovine serum was added to the medium.

2.8 Culture and cryopreservation of bacteria and fungus

All bacteria and fungus were cultured on tryptone soy agar (Appendix 1) and Sabouraud (Appendix 1) plates respectively. Plates were then incubated at 37°C for 48 hrs. For cryopreservation, several colonies of bacteria or fungus were scraped using sterile disposable loop and suspended in ¼ strength Ringer's solution (Appendix 1). The cells were then centrifuged at 5000 × *g* for 1 min. Pellet was resuspended in cryoprotectant containing 95% (v/v) heat-inactivated foetal bovine serum and 5% (v/v) DMSO. Suspension was then aliquoted into cryotubes and kept at -80°C for long term storage. Recovery of the cells was done by scraping the ice culture using a sterile disposable loop prior streaking on an appropriate medium as described earlier.

2.9 Cultures of mammalian cell lines

The cell lines used (MA104, Hep-2 and EA.hy926) were grown in Nunc tissue culture flasks (Fisher Scientific, Loughborough, UK) at 37 °C with 5% (v/v) CO_2. The medium

used was Dulbecco's Modified Eagle Medium supplemented with 2 mM L-glutamine and 10% (v/v) heat-inactivated foetal bovine serum.

For drug sensitivity assays, the MA104 cells were grown in a 96-well plate. Briefly 5×10^4 cells/ml was seeded into each well containing growth medium. Plate was incubated as described above.

2.9.1 Subculturing cell lines

All cell lines were subcultured once a week. The spent medium used to grow the cells was removed and monolayer was washed with 5-10 ml of Dulbecco A phosphate buffered saline (DPBS, Appendix 1). The DPBS was then discarded and 2-4 ml of TrypLE Express was added to each flask and incubated for 5-10 min at RT to allow detachment of cells from the flask's surface. This could be observed using an inverted microscopy. The flask was gently tapped at the side to completely detach the cells prior to subculture into 3 to 4 new flasks containing growth medium as described in Section 2.9. The flasks were then incubated at 37°C with 5% (v/v) CO_2.

2.10 Culture of *B. mandrillaris*

MA104 cells were initially grown as described in Section 2.9. Once a monolayer was formed, the medium were discarded and rinsed with DPBS (Appendix 1). Fresh maintenance medium containing DMEM with 2 mM L-glutamine was added, followed by *B. mandrillaris*. The flask was incubated at 37°C with 5% (v/v) CO_2. The clearance of the monolayer and the presence of *B. mandrillaris* trophozoites were observed using an inverted microscope.

2.10.1 Subculturing of *B. mandrillaris* culture

Balamuthia mandrillaris fed with MA104 cells was subcultured once a week. Briefly, flask was checked using microscopy for clearing of monolayer and presence of *B. mandrillaris*. The flask was gently tapped at the side to detach the amoebae from the flask surface. *B. mandrillaris* was harvested by centrifugation at 800 × *g* for 3 min. The pellet was resuspended in 2 ml of maintenance medium (Section 2.10) and a cell count was performed using a modified Fuchs Rosenthal haemocytometer. *B. mandrillaris* was then transferred onto a fresh MA104 monolayer at a seeding concentration of 1-2 × 10^4 *B. mandrillaris*/ml.

2.11 Cryopreservation of axenic amoebae and cell lines

For cell lines, once the monolayer was formed the culture medium was discarded. The monolayer was rinsed with DPBS (Appendix 1) and 2 ml of TrypLE Express was added. The flask was left for 5-10 min at RT to allow the cells to detach from the flask's surface. A volume of 8 ml of maintenance medium (DMEM with 2 mM L-glutamine) was added to the flask to resuspend the detached cells. The suspension was then centrifuged at 800 × *g* for 3 min. The pellet was then resuspended in cryoprotectant containing 50% (v/v) FBS, 40% (v/v) DMEM with 2mM L-glutamine and 10% (v/v) DMSO and aliquoted into cryotubes.

For axenic cultures, including of *Acanthamoeba* spp., *Naegleria* spp., and *B. mandrillaris*, amoebae were initially grown in the appropriate medium. Following that, amoebae were harvested by centrifugation at 800 × *g* for 3 min. The pellet was resuspended in cryoprotectant containing 50% (v/v) FBS, 40% (v/v) appropriate medium and 10% (v/v) DMSO and aliquoted into cryotubes. All cryotubes were then placed in a Nalgene Cryo 1°C freezing container (Fisher Scientific, Loughborough, UK)

that had been filled with propan-2-ol. The propan-2-ol functioned to minimise cellular damage by controlling the rate of temperature decrease to 1°C/min. The container was then kept in a -80°C freezer (Sanyo Gallenkamp, Loughborough, UK) for 3 days prior storage in liquid nitrogen tank.

2.11.1 Recovery of cryopreserved amoebae and cell lines

Cryotubes were removed from liquid nitrogen and the contents were thawed in a 37°C waterbath. Appropriate culture medium was prepared and aliquoted into tissue culture flasks. For recovery of *B. mandrillaris*, MA104 monolayer was grown in a flask as described in Section 2.9. Thawed cells were then transferred into the appropriate flask and incubated at the appropriate temperature. Culture medium was replaced with fresh medium after 12 hours of incubation to reduce the toxicity caused by the DMSO.

2.12 Culturing *B. mandrillaris* on different cell lines

Monolayers of MA104, Hep-2 and EA.hy926 cells were grown in 25 cm^2 tissue culture flasks, as described in Section 2.9. The culture medium was discarded and rinsed with DPBS (Appendix 1). About 1×10^4/ml of *B. mandrillaris* was then added to each of the flask containing fresh maintenance medium (DMEM and 2mM L-glutamine) and incubated at 37°C with 5% (v/v) CO_2. The presence of amoebae and clearing of monolayer were observed daily using an inverted microscopy (CKX41, Olympus). The *B. mandrillaris* cell count was performed using a modified Fuchs-Rosenthal hemocytometer once the monolayer had been cleared by the amoeba. Experiment was conducted in triplicate.

2.13 Preparation of NNA and NNA-*E. coli* plates lawned with free-living amoebae

Acanthamoeba castellanii (ATCC 50370) and *Naegleria gruberi* (ATCC 30224) were initially grown as described in Sections 2.3 and 2.7. Amoebae were diluted in DPBS (Appendix 1) to a final concentration of 1×10^6/ml. NNA and NNA-*E. coli* plates (Appendix 1 and Section 2.5) were then lawned in triplicate using a sterile hockey shaped spreader with 1) suspension of *A. castellanii* alone, 2) suspension of *N. gruberi* alone and 3) suspension with a mixture of both amoebae. The amoebae were then allowed to settle at RT for 30 min prior to use.

2.14 Preparation NNA plates lawned with bacteria and fungus

Four bacteria [*Bacillus* sp. (JPN), *Escherichia coli* (JM101), *Staphylococcus aureus* (ATCC 6538) and *Stenotrophomonas maltophilia* (ATCC 15099)] and the fungus *Candida albicans* (ATCC 10231) were grown as described in Section 2.8. About 10 colonies were scraped from each plate with a disposable loop and resuspended in sterile tubes containing 2 ml of nanopure water. The suspension of bacteria or fungi, as required by the experiment was then lawned on NNA plates (Appendix 1) in triplicate using a spreader. Plates were left to dry at RT for 30 min.

2.15 Culture of *B. mandrillaris* on NNA and NNA-*E. coli* plates lawned with free-living amoebae, bacteria and fungus

A 30 µl suspension containing 1×10^5 of *B. mandrillaris* was put at the edge of the NNA and NNA-*E. coli* plates that had been lawned with free-living amoebae, bacteria and fungus (Sections 2.13 and 2.14). The area was circled with a permanent marker. Plates were then sealed with parafilm and incubated for up to 12 days at 25°C and were

checked regularly, using an inverted microscope, for the migration of *B. mandrillaris* from the circled area. The distance of migration was measured using a ruler and recorded. All experiments were conducted in triplicate.

2.16 Environmental samples

A total of 186 environmental samples were used in the study of the ecology of the FLA. The details are shown in Table 2.2. The pH was determined from all environmental samples, except those from France, using Whatman pH 1-11 indicator paper (VWR Int. Ltd., Lutterworth, UK). Other parameters, including dissolved oxygen (Section 2.16.1), conductivity and temperature (Section 2.16.2), were only performed with water samples collected in the UK.

2.16.1 Dissolved oxygen

Dissolved oxygen was measured using an O2 test kit (Salifert, Tropical Marine Centre, Chorleywood, UK). Briefly, 5 ml of water sample was added to the test vial provided. In the same vial, 5 drops of O2-1 solution was added and swirled gently for 20 sec. Six drops of O2-2 solution was then added and swirled again for 15 sec and allowed to stand for 1 min. Following that, 6 drops of O2-3 solution was added and swirled for 20 sec. Finally test vial was allowed to stand for 30 sec for colour development. The test vial was placed on a white paper and the colour produced was compared with the colour chart provided.

2.16.2 Conductivity and temperature

Conductivity was measured using HI 8733 conductivity meter (Hanna instruments Ltd., Leighton Buzzard, UK). The probe was submerged into the water sample and the reading displayed on the screen was recorded. Temperature was measured with HI 93531 portable thermometer (Hanna instruments Ltd., Leighton Buzzard, UK).

Table 2.2 Details of environmental samples used in the study of ecology of the FLA

Location/Provider	Latitude/longitude	Material	No. of samples
River Tarn, South West France[a]	44° 6' 24" N, 0° 50' 43" E	Water	109
River Trent, Nottinghamshire, UK[b]	52° 51′ 55″ N, 1° 15′ 18″ W	Water	10
Leicestershire[b] and Reading[c], UK	51° 28' 22" N, 0° 54' 13" W and 52° 38′ 3″ N, 1° 8′ 19″ W	Soil	34
Southern California, USA[d]	33° 44' 2.2" N, 117° 47' 49.3" W and 33° 42' 33.1" N, 117° 53' 34.5" W	Soil	17
Kruger Park and Cape Town, RSA[e]	24° 0' 41" S, 31° 29' 7" E and 33° 55' 31" S, 18° 25' 26" E	Soil	2
Poggibonsi, Italy[f]	43° 28′ 0″ N, 11° 9′ 0″ E	Soil	6
Loule, Portugal[g]	37° 9' 0" N, 8° 0' 0" W	Soil	8

[a] = Dr. James Lonnen, University of Leicester, UK

[b] = Arine Fadzlun Ahmad, University of Leicester, UK

[c] = Dr. Elizabeth Jane Shaw, University of Reading, UK

[d] = Dr. Simon Kilvington, Abbott Medical Optics Inc., USA

[e] = Dr. Wayne Heaselgrave, University of Leicester, UK

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2.17 Cultivation of environmental samples on NNA-*E. coli* plates

Aliquots of the resuspended pellet of water samples obtained by centrifugation (Section 2.18.4.1) or filtration (Section 2.18.4.2) were inoculated as five small drops onto NNA-*E. coli* plates (Section 2.5) and incubated at 32°C and 44°C for up to 7 days. In addition, aliquots of soil suspensions prepared in nanopure water were also cultured on NNA-*E. coli* plates. Plates were examined daily using an inverted microscope for growth of amoeba. Any areas that were suspected of containing amoebae were streaked out using a sterile tooth pick and inoculated into wells of a microtitre plate containing 3-4 drops of ¼ of Ringer's solution, prior incubation at 32°C. The morphology of the amoebae was then confirmed by microscopy. The ability of the amoebae to form a flagellate stage was also determined.

2.18 Extraction of deoxyribonucleic acid (DNA) from environmental samples

2.18.1 UNSET-PEG method (small-scale soil samples)

One gram of soil was weighed and transferred into a 2 ml screw-capped microcentrifuge tube (Sarstedt, Leicester, UK) containing 0.75 g of 0.25 - 0.5 mm glass beads (Jencons, West Sussex, UK). One ml of UNSET lysis buffer (Appendix 1) (Hugo *et al.*, 1992) was added and the soil completely resuspended by scraping with a loop. Subsequently, the suspension was processed either by using a ribolyser or vortex (Section 2.18.1.1) and tubes containing the suspension were centrifuged at 10,000 × *g* for another 5 min. One ml supernatant from each tube was transferred to a 1.5 ml microcentrifuge tube and 0.5 ml 30% (w/v) PEG 6000 in 1.6 M NaCl (Appendix 1) was added. The solution was mixed thoroughly by inversion and incubated at RT for two hours with occasional mixing (or overnight at 4°C). The suspension was further centrifuged at 10,000 × *g* for

another 20 min and pellet was dissolved in 1 ml TE buffer (10 mM Tris-HCl, 1mM sodium EDTA, pH 8.0) using a sterile Pasteur pipette. Potassium acetate (7.5 M) was added to give a final concentration of 0.5 M and the suspension was placed on ice for 5 min prior centrifugation at 12,000 × *g* for 30 min at 4°C (Yeates *et al.*, 1997), to precipitate proteins and polysaccharides. The supernatant was transferred into a new 1.5 ml microcentrifuge tube and further extracted twice with phenol:chloroform (1:1) and once with chloroform for protein removal. The supernatant was collected and 0.8 volumes of ice-cold isopropanol was added, mixed by inversion and kept at 4°C overnight. Subsequently, the suspension was centrifuged again at 10,000 × *g* for 15 min and pellet was washed twice with 70% (v/v) ethanol. Excessive alcohol was aspirated by pipetting and pellet was left to dry at 44°C for 30 min. Finally the dried pellet was dissolved in 50-100 µl of TE $_{0.1mM}$ (10 mM Tris-HCl, 0.1mM sodium EDTA, pH 8.0) and kept at 4°C or 20°C for longer storage.

2.18.1.1 Mechanical cell disruption

Two methods were used to disrupt the cells contained in the soil samples. The first method involved the use of a ribolyser (Hybaid, Sharpedge Engineering Ltd., Newbury, UK) for 30 seconds at power 5.5 while the second method by vortexing (Camlab, Cambridge, UK) for 30 seconds.

2.18.2 DNA purification

2.18.2.1 Chelex method

An equal volume of Chelex solution (Appendix 1) was mixed with crude DNA (Section 2.18.1) by vortexing for 5 sec. The suspension was then centrifuged at 10,000 *g* for 10 sec. The tube containing the suspension was heated at 95°C for 15 min and centrifuged at 10,000 *g* for 10 sec. The supernatant was retained and stored at 4°C.

When DNA was extracted from amoeba cultures, the pellet of amoeba was dissolved in 200 µl of Chelex solution in a sterile 0.5 ml microcentrifuge tube and processed as above.

2.18.2.2 Yorbio gel/ PCR DNA purification kit

The kit was purchased from Yorkshire Bioscience Ltd., York, UK. Equal volumes of binding buffer and crude DNA were transferred into a 1.5 ml microcentrifuge tube, vortexed and mixed by pipetting. The mixture was then incubated at room temperature for 1 min and transferred to a spin column with a 2 ml collection tube and left to stand for 2 min. Subsequently, the spin column containing the mixture was centrifuged for 1 min and the eluate was discarded. All centrifugation steps were done using a benchtop microcentrifuge (Eppendorf Ltd., Cambridge, UK) at 10,000 × *g*. The spin column was then washed twice by centrifugation for 15 sec each with wash solution supplied by the manufacturer. The column was put into a new 2 ml collection tube and centrifuged for another 1 min to remove the residual washing solution. Finally, the column was transferred into a sterile 1.5 ml microcentrifuge tube and 60 µl of elution buffer was added onto the centre part of the column membrane, incubated for 2 min at room temperature and centrifuged for 1 min to elute the purified DNA.

2.18.2.3 ZR soil microbe DNA kit

The kit was a product of Zymo Research Corp., USA and purchased from Cambridge Bioscience, Cambridge, UK. Steps 1-3 from the manual that involved cells disruption using a bead-beater were omitted. Briefly, the base of a Zymo-Spin IV spin filter tube in a 2 ml collection tube was snapped off prior use, crude DNA added and centrifuged at 7,000 × *g* for 1 minute. A volume of 1.2 ml of DNA binding buffer was added to the filtrate in the collection tube and mixed by pipetting. Half of the mixture was transferred

to a Zymo-Spin IIC column in a collection tube and centrifuged at 10,000 × *g* for 1 min. The eluate was discarded and the previous step was repeated using the other half of the mixture. A volume of 200 µl DNA pre-wash buffer was added to the Zymo-Spin IIC column in a new collection tube and centrifuged again for another 1 min at the same speed. Subsequently, 500 µl of soil DNA wash buffer was added to the column and centrifuged for 1 min. The column was then transferred into a clean 1.5 ml microcentrifuge tube and 60 µl of DNA elution buffer was added directly to the column matrix and centrifuged at 10,000 × *g* for 30 seconds to elute the DNA. Next, the Zymo-Spin IV-HRC spin filter tube was prepared by snapping of the base, inserting into a collection tube and centrifuged for 3 min at 8,000 × *g*. The tube was then transferred into a sterile 1.5 ml microcentrifuge tube, added with eluted DNA and centrifuged for another 1 min at 8,000 × *g* to collect the purified DNA.

2.18.3 UNSET-PEG method (large-scale soil samples)

Soil samples were processed as described in Section 2.18.1 but with some modifications. Ten grams of soil sample was used for each DNA extraction process. The soil sample was transferred into a 50 ml Corning centrifuge tube (Fisher Scientific, Loughborough, UK) containing 4 g of 0.25 – 0.5 mm glass beads prior to the addition of equal volume (10 g = 10 ml) of UNSET lysis buffer. All centrifugation steps were done at 3900 × g and the time for each step was twice that mentioned previously (Section 2.18.1) with the exception for centrifugation step at 4°C which was done for 30 min. The volumes for all solutions used were also increased proportionally for those described in Section 2.18.1. Mechanical cell disruption was performed using a vortex for 1 min followed by 30 min incubation in UNSET solution. The final pellet was dissolved in 50-100 µl of TE $_{0.1mM}$ (10 mM Tris-HCl, 0.1mM sodium EDTA, pH 8.0).

Crude DNA obtained was finally purified with ZR soil microbe DNA kit (Section 2.18.2.3).

2.18.4 Extraction of DNA from water samples

Depending on the turbidity of the water samples, processing was done either by centrifugation (Section 2.18.4.1) or filtration (Section 2.18.4.2). The methods used for DNA extraction were the same as described in Section 2.18.3.

2.18.4.1 Centrifugation

Seven hundred and fifty millilitres of water sample containing sediment was centrifuged at 3000 × *g* for 30 min using an Avanti J-E floor base centrifuge (Beckman Coulter Ltd., High Wycombe, UK). The supernatant was discarded and the pellet was transferred into a 50 ml centrifuge tube and centrifuged again at 1000 × *g* for 10 min. The supernatant was discarded, leaving approximately 1.5 ml and the pellet was resuspended by vortexing for 5 sec. DNA extraction and purification were performed as described in Sections 2.18.3 and 2.18.2.3.

2.18.4.2 Filtration

All water samples without sediment were processed simultaneously by manifold filtration. Initially, bottles containing water samples were shaken vigorously to mix the contents. A volume of 750 ml of each water sample was then filtered through a 0.45 µm pore size cellulose nitrate membrane (Sartorius Ltd., Surrey, UK) without allowing the membrane to run dry. Filtration was stopped when approximately 10 ml of water remained above the membrane. The residual water above the membrane and the membrane was transferred into a universal tube with the surface containing filtered materials facing inwards from the walls of the tube. The container was then vortexed for 10 sec and the material trapped on the membrane was scraped with a sterile cotton-

tipped swab and expressed into the tube. The membrane was then removed and the tube centrifuged at 1000 × *g* for 10 min. Pellet was resuspended with 1.5 ml of the supernatant and DNA extraction and purification were performed as described in Sections 2.18.3 and 2.18.2.3.

2.18.5 Determination of DNA concentration

The concentration of purified DNA was determined either using a Nano-drop ND-1000 spectrophotometer, A_{260nm} (Labtech International, East Sussex, UK) or by comparison of the DNA intensity with a known amount of DNA marker after agarose gel electrophoresis (Section 2.18.6).

2.18.6 Agarose gel electrophoresis

Agarose gel electrophoresis was done to observe the presence of DNA that has been extracted and amplified after polymerase chain reaction (PCR). A volume of 5 µl and 10 µl of crude or purified DNA and PCR products respectively were subjected to electrophoresis through 1.5% (w/v) agarose gels that contained ethidium bromide (0.5 µg/ml) and visualised under ultraviolet (UV) transilluminator (3UV Transluminator, LMS-20E, UVP).

2.18.6.1 Casting 1.5% (w/v) agarose gel

Agarose MP powder (Roche Ltd, Burgess Hill, UK) (1.5 g) was transferred into an Erlenmeyer flask containing 100 ml of 1× Tris-acetate EDTA (TAE) buffer (Appendix 1). The mixture was then heated in a microwave oven until the agarose has dissolved. Subsequently, the dissolved agarose was allowed to cool to 55ºC by placing the flask into a container with running tap water. After the molten gel had cooled, ethidium bromide (10 mg/ml, Promega, Southampton, UK) was added to produce a final concentration of 0.5 µg/ml. The mixture was mixed thoroughly by gentle swirling

before being poured into a horizontal gel casting tray (Owl) with the appropriate comb in place.

Care was taken to ensure that the agarose gel covered about one-third the height of the comb teeth or a depth of about 5 mm. A pipette tip was used to remove bubbles or solid debris to the sides or end of the tray while the gel was still in liquid form. The gel was then allowed to solidify at room temperature. After the gel had set, the comb was removed carefully and the tray was placed into a horizontal tank with the wells oriented at the cathode end. A sufficient volume of 1×TAE buffer was poured into the tank to cover the agarose gel.

2.18.6.2 Electrophoresis of DNA and PCR products

A droplet (1 µl) of 6X bromophenol blue (Fermentas, York, UK) loading dye solution was placed onto a small square of Parafilm. Five µl of crude or purified DNA was withdrawn from the microcentrifuge tube and mixed with the loading dye droplet by pipetting in and out to produce a mixture with a volume of 6 µl each. Subsequently, the mixture was loaded into the well of the agarose gel prepared as in Section 2.18.6. Alternatively, 10 µl of each PCR product were loaded directly into wells. A new sterile pipette tip was used for loading each DNA and PCR samples. A standard marker was included in every electrophoresis by using a 100 bp or 1 kb DNA ladder (Fermentas, York, UK).

The lid of the gel tank was closed and an electrical voltage of 90 V was applied for 45 to 60 minutes. After the marker dye had migrated to a sufficient distance through the gel (about 7 cm from the well), the electrophoresis was stopped. The gel was removed from the tank and was ready to be examined under medium-wavelength ultraviolet (UV) light (UV-B, 302 nm). Exposure of DNA to UV light was kept to minimum. The gels were

photographed using a gel documentation device (DOC-088.XD, UVItech) and saved in a digital JPEG format.

2.19 Polymerase chain reaction (PCR)

A volume of 1-4 µl of purified DNA was subjected to amplification using primers shown in Table 2.3.

Table 2.3 Primers used in this study

Primers	Sequences
Modified prokaryotic 16S rDNA (Fierer *et al.*, 2005, Nadkarni *et al.*, 2002)	**338F** 5'- ACTCCTACGGGNGGCNGCA-3' **797R** 5'- GGACTACCAGGGTATCTAATCCTGTT-3'
External *B. mandrillaris* (ExtF/R)	**ExtF** 5'-GGTTCGTGCCCCTTGCCTTCTG-3' **ExtR** 5'-CGACTTTGCCCAACCTCACGAC-3'
Internal *B. mandrillaris* (IntF/R)	**IntF** 5'-CCACACCTTGGCCG-3' **IntR** 5'-GGTCGAGCTCCGAA-3'
Nuclear 18S rDNA *B. mandrillaris* (Qvarnstrom *et al.*, 2006)	**BalaF1451** 5′-TAACCTGCTAAATAGTCATGCCAAT-3′ **BalaR1621** 5′-CAAACTTCCCTCGGCTAATCA-3′
Mitochondrial 16S rDNA *B. mandrillaris* (Booton *et al.*, 2003b)	**5′Balspec16S** 5'-CGCATGTATGAAGAAGACCA-3′ **3′Balspec16S** 5′-TTACCTATATAATTGTCGATACCA-3′
Outer *N. fowleri* (OP4F/R)	**OP4F** 5'-GCCTTTCTTCGGCTCGCATG-3' **OP4R** 5'-CTTGAGTGCACGCCACTTGAT-3'
Internal *N. fowleri* (IP4F/R)	**IP4F** 5'-CAGGAATGTCATCACAC-3' **IP4R** 5'-GAATGAGTACTCGTTGC-3'
N. fowleri (NF2F/R, in-house)	**For** 5'-TACTGGCTCGAGTGTGATGG-3' **Rev** 5'-GAGGAGCAGTCGATGATTGG-3'
N. lovaniensis (NL5F/R, in-house)	**For** 5'-TGGTACAAGACACCCAGATAGG-3' **Rev** 5'-CTTGGTGTCCAAACTCCTCAG-3'
Acanthamoeba 18S rDNA (Schroeder *et al.*, 2001)	**JDP1** 5'-GGCCCAGATCGTTTACCGTGAA-3' **JDP2** 5'-TCTCACAAGCTGCTAGGGGAGTCA-3'.

2.19.1 PCR to detect prokaryotes

A modified prokaryotic 16S rDNA primer set (Table 2.3) was used to amplify DNA of bacteria to give a PCR product of approximately 500 bp. PCR was performed in a volume of 20 µl containing 2X ReddyMix PCR Master Mix (0.625 U Thermoprime Plus DNA polymerase, 75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 1.5 mM MgCl2, 0.01% (v/v) Tween 20, 0.2 mM dNTPs) (ABgene, Surrey, UK), 0.5 µM of each primer and sterile nanopure water. The programme used was a pre-PCR heat cycle at 95ºC for 5 min, followed by 30 cycles at 94ºC for 30 sec, 60ºC for 30 sec, 72ºC for 40 sec, and an extension at 72ºC for 10 min. *Escherichia coli* strain JM101 DNA (1 µl) was used as the positive control. Those environmental samples which gave negative results were re-amplified (1 µl) using the same primer set. For the negative control, the DNA template was replaced with the same volume of distilled water.

2.19.2 Detection of *B. mandrillaris* in soil and water samples

Two sets of primers were specifically designed for amplifying DNA of *B. mandrillaris* from environmental samples by a nested PCR. Partial 18S rDNA sequences of *B. mandrillaris* (AF477019, AF477020, AF477021, AF477022 and AF019071) were retrieved from the GenBank and aligned using CLUSTALW software (Chenna *et al.*, 2003). The conserved regions among the isolates were selected and similarity search analyses were performed using the basic local alignment search tool (BLAST) software (Altschul *et al.*, 1990). The external and internal primers (refer Table 2.3) were expected to give PCR products of 403 bp and 201 bp respectively. PCR volume was the same as those for prokaryotic 16S rDNA amplification (Section 2.19.1). Reaction conditions for both set of primers were the same, except for the annealing temperature. A pre-heat cycle at 95°C for 5 min was followed by 30 cycles at 94°C for 30 sec, 56°C (ExtF/R) or 48°C (IntF/R) for 30 sec, 72°C for 30 sec and an extension at 72°C for 10

min. Samples were initially subjected for primary PCR with the external *B. mandrillaris* primers (ExtF/R). Following that 1µl of the PCR product was used as the DNA template for the subsequent PCR using IntF/R primers.

Selected environmental samples were also tested for PCR amplification using published *B. mandrillaris* nuclear 18S rDNA and mitochondrial 16S rDNA primers (Table 2.3). The PCR using the published *B. mandrillaris* nuclear 18S rDNA primer set was performed in a final volume of 20 µl containing 2X ReddyMix PCR Master Mix (ABgene, Surrey, UK), 0.2 µM of each primer and sterile nanopure water. The reaction condition was a pre-heat cycle at 95°C for 5 min and 40 cycles at 95°C for 30 seconds, 56°C for 30 sec, 72°C for 30 sec and a final extension at 72°C for 10 min.

The PCR using the published *B. mandrillaris* mitochondrial 16S rDNA primer set was performed in a volume of 50 µl containing 2X ReddyMix PCR Master Mix, 2 µM of each primer and nanopure water. The PCR reaction was 40 cycles of 94°C for 1 min, 48°C for 2 min, 72°C for 3 min and a final elongation step at 72°C for 15 min.

2.19.2.1 Specificity and sensitivity tests for the *Balamuthia* nested PCR primers

The developed *B. mandrillaris* nested PCR primers were tested against DNA of other prokaryotes and eukaryotes as listed in Table 2.3. For sensitivity testing, *B. mandrillaris* genomic DNA was diluted in DPBS (10 ng-0.01 pg) and subjected for nested PCR amplification.

DNA of spiked soil samples used in the sensitivity test were prepared by adding 10^1 to 10^3 *B. mandrillaris* trophozoites to 10 g of soil sample (EM08-03) and DNA was extracted as described in Section 2.18.3. The EM08-03 soil sample was initially tested to be *Balamuthia*-negative by the nested PCR.

2.20 Extraction and purification of *Naegleria fowleri* plasmid DNA

LB-Amp plates were initially prepared for growing *E. coli*. LB agar (Appendix 1) was prepared and autoclaved. The broth was left to cool to about 55°C. Then ampicillin stock (100 mg/ml) was added to give a final concentration of 100 µg/ml and contents were swirled and poured into Petri dishes. The contents were allowed to harden at RT and kept at 4°C until used. A loopful of *E. coli* was streaked onto the LB-Amp plates and incubated at 37°C for overnight. The next day, one colony of bacteria was added into 5 ml of LB-Amp broth and kept in shaking incubator rpm 100 rpm 30°C, overnight.

DNA was extracted using a Zyppy Plasmid Miniprep kit (Zymo Research). The overnight bacteria culture was centrifuged at 1000 × *g* for 3 min and resuspended in 600 µl TE buffer. A volume of 100 µl of 7× lysis buffer was added and mixed by inverting the tube 4-6 times. A complete lysis was shown by change in the appearance from opaque to clear blue. Immediately (within 2 minutes) 350 µl cold neutralisation buffer was added and mixed thoroughly. The tube was inverted for another 2-3 times to ensure complete neutralisation and centrifuged at 16,000 × *g* for 4 min. All subsequent centrifugations were done at this speed. The supernatant was transferred into a Zymo-Spin IIN column without disturbing the cell debris pellet. The column was placed into a collection tube and centrifuged for 15 sec. Flow-through was discarded and the column was placed back into the same collection tube. A volume of 200 µl Endo-Wash Buffer was added to the column and centrifuged for 15 sec. This was followed with the addition of 400 µl Zyppy Wash buffer to the column and centrifuged for 30 sec. The column was then transferred into a clean 1.5 ml microcentrifuge tube and 30 µl Zyppy Elution Buffer was added directly to the column matrix. The tube was allowed to stand at RT for 1 min prior to centrifugation for 15 sec to elute the plasmid DNA.

2.20.1 Detection of *Naegleria fowleri* from water samples by one-step nested PCR

A one-step nested PCR was utilised for the detection of *Naegleria fowleri* from water samples. The primers (Table 2.3) were developed from a cloned fragment of *N. fowleri* (MCM) DNA (pUC PB2.3), shown from hybridization and PCR analysis to be specific for the organism (Kilvington *et al.*, 1995b, Kilvington *et al.*, 1995a). The PCR products for OP4F/R and IP4F/R were 767 bp and 506 bp respectively. PCR was performed in 40 µl volume containing 2X ReddyMix PCR Master Mix (0.625 U Thermoprime Plus DNA polymerase, 75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 1.5 mM MgCl2, 0.01% (v/v) Tween 20, 0.2 mM dNTPs) (ABgene, Surrey, UK), 0.1 µM of each outer primer and 0.5 µM of each internal primer. The reaction condition used was a pre-heat at 94°C for 4 min, 20 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1min, 35 cycles of 94°C for 1 min, 48°C for 1 min, 72°C for 1min and a final elongation step at 72°C for 10 min.

2.20.1.1 Sensitivity and specificity of *N. fowleri* nested PCR

Sensitivity testing was performed using purified *N. fowleri* DNA (KUL, ATCC 30808) that had been diluted in Dulbecco A phosphate buffered saline (Appendix 1) to form concentrations of 1ng/µl, 100 pg/µl, 10 pg/µl, 1 pg/µl and 100 fg/µl.

Specificity testing was performed using DNA of *E.coli* (JM101), *A. castellanii* (ATCC 50370), *A. polyphaga* (CCAP 1501/3G), *B. mandrillaris* (ATCC 50209), *N. gruberi* (ATCC 30224), *A. polyphaga* (ATCC 30461), *Acanthamoeba* sp. (Ros), *A. hatchetti* (CDC V573), *A. castellanii* (ATCC 30234), *Acanthamoeba* spp. (SCSK09-01 to SCSK09-07), *N. lovaniensis* (C0490), *N. fowleri* (CCAP 1518), *N. fowleri* (KUL, ATCC 30808), *N. fowleri* (NH1), *N. fowleri* (MSM) and *N. fowleri* plasmid DNA (pUC PB2.3).

N. lovaniensis-*N. fowleri* duplex PCR primers (NF2F/R, NL5F/R, Table 2.3) were used to confirm the identity of *N. lovaniensis* strain C0490. PCR was performed separately using primer set NL5F/R (*N. lovaniensis*) and NF2F/R (*N. fowleri*) as individual pairs and a combination of both set of primers. The PCR was performed in a final volume of 40 µl containing 2X ReddyMix PCR Master Mix, 0.5 µM (NF2F/R) or/and 0.25 µM (NL5F/R) of each primer set and nanopure water. The condition used was a pre-heat cycle at 95°C for 5 min and 35 cycles at 94°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec and a final elongation step at 72°C for 10 min.

2.20.2 Detection of *Acanthamoeba* sp. from soil samples

Purified DNA from soil samples were also subjected for PCR amplification using *Acanthamoeba* 18S rDNA primers (Schroeder *et al.*, 2001). Amplification reactions were performed in a volume of 20 µl containing 2X Reddy Mix PCR Master Mix, 0.5 µM of each primer and nanopure water. The PCR condition was a pre-PCR heat cycle at 95 ºC for 5 min, followed by 40 cycles at 94 ºC for 30 sec, 60 ºC for 30 sec, 72 ºC for 30 sec, and an extension at 72 ºC for 10 min.

2.20.3 Sequencing of PCR products

Sequencing of PCR products was performed at Eurofins MWG Operon, London, UK. For this, the selected PCR product was first purified using NucleoSpin Extraction II (Macherey-Nagel, Fisher Scientific, Loughborough, UK). DNA fragments were excised from the agarose gel was excised using a clean and sterile scalpel. The gel slice was weight and transferred into a sterile 2 ml tube. For each 10 mg of agarose gel, 20 µl of buffer NT was added. The mixture was then incubated at 50°C for 5-10 min with occasional vortexing or until the gel was completely dissolved. A NucleoSpin Extract II column containing silica membrane was then placed into a 2 ml collection tube supplied and the dissolved gel was loaded. The tube, together with the column, was then

centrifuged for 1 min at 11,000 × *g* to bind the DNA on the silica membrane. The flow-through was discarded and the column was placed back into the 2 ml collection tube. The trapped DNA was then washed by adding 600 µl of buffer NT3 onto the column and centrifugation for 1 min at 11,000 × *g*. Flow-through was discarded and a second washing step was done by adding 200 µl of buffer NT3 and centrifugation at the same speed for 2 min. Carefully, the column was removed and placed into a clean 1 ml microcentrifuge tube. A volume of 35 µl of elution buffer NE was added onto the center of the column and incubated at RT for 1 min. The DNA was eluted by centrifugation at 11,000 × *g* for 1 min. The purified DNA was then sent for sequencing using the IntR primer for *B. mandrillaris* and IP4R primer for *N. fowleri*. DNA sequences results were finally retrieved from the company website or by email. Subsequently, the quality (clipped) DNA sequences of *B. mandrillaris* and *N. fowleri* were analysed against all possible DNA sequences in the GenBank and the cloned *N. fowleri* DNA sequence respectively using BLAST software (Altschul *et al.*, 1990).

2.21 Preparation of *B. mandrillaris* extract for use as an immunogen

B. mandrillaris was initially cultured as described in Section 2.10. Once the monolayer has been cleared by the *B. mandrillaris*, the trophozoites were detached from the flask surface by gentle tapping and harvested by centrifugation at 800 × *g* for 3 min. The trophozoites were washed twice with Dulbecco A phosphate buffered saline (DPBS) by centrifugation and pellet was resuspended in small amount of DPBS and kept at -20°C prior to sonication or immediately formalin-treated. Details of each method are described below.

2.21.1 Sonication

The suspension of *B. mandrillaris* was thawed and kept on ice to reduce protein degradation. Sonication was done at amplitude of 7 microns, using a MSE Soniprep 150 (Wolf Labs, York, UK). The sonication was carried out for 20 seconds with intervals 20 seconds to avoid excessive heating of the probe and sample. This sequence was repeated 10 times to ensure all trophozoites were totally lysed. The suspension was then centrifuged for 15 min at 3500 × *g* and the supernatant was aliquoted and stored at -20°C.

2.21.2 Formalin treatment

B. mandrillaris trophozoites were harvested as described in Section 2.21. The pellet was then resuspended with cold 0.5% (v/v) formalin in DPBS by gentle pipetting and incubated overnight at 4°C. The pellet was then washed twice with 10 ml of DPBS by centrifugation at 500 × *g* for 1 min, 4 °C to remove any residual formalin. Pellet was resuspended with cold DPBS and keep at -20°C until used.

2.22 Production of *B. mandrillaris* polyclonal antibodies in mice

This was done by Dr. Hassan Yesilkaya from Dept. of Infection, Immunity and Inflammation, University of Leicester. Three 10 weeks old female MFI mice (Harlan Olac, UK) were injected with a 100 µl containing 25 µg of sonicated amoebae in 33 µl of Imject Alum adjuvant (Perbio Science, Cramlington, UK) and 67 µl of DPBS. As a control, mice were immunised with adjuvant and DPBS only. Injections were repeated fortnightly three times. Two weeks after the final immunisation, mice were anesthetised with 5% (v/v) fluothane (Astra Zeneca, Macclesfield, UK) over oxygen (1.5 to 2 l min^{-1}) and blood was collected by cardiac puncture and then the animals were killed by cervical dislocation without allowing them to recover from anaesthesia. The blood was

left to clot for 1 hour at room temperature. Finally, the serum containing polyclonal antibodies against *B. mandrillaris* was recovered by centrifugation at 5000 × *g* for 10 minutes and stored at -80°C until used.

2.23 Production of *B. mandrillaris* polyclonal antibodies in rabbits

Production of *B. mandrillaris* polyclonal antibodies was done in two rabbits (UK4237 and UK4238) by Cambridge Research Biochemicals, Cleveland, UK using formalin-treated *B. mandrillaris* trophozoites. The route of immunisation was subcutaneous dorsal. All immunisations were performed with Freund's incomplete adjuvant, except for the first immunisation which used Freund's complete adjuvant. Samples of the serum from each pre-bleed and test bleed was analysed by ELISA (Section 2.28) to determine the *B. mandrillaris* antibody levels in both rabbits. The immunisation schedule is in Table 2.4.

Table 2.4 Immunisation schedule for production of *B. mandrillaris* polyclonal antibodies in rabbits

Procedure	Day
Pre-immune bleed and immunisation 1	0
Immunisation 2	14
Test bleed 1	21
Immunisation 3	28
Test bleed 2	35
Immunisation 4	42
Test bleed 3	49
Immunisation 5	56
Test bleed 4	63
Immunisation 6	70
Test bleed 5	126
Harvest bleed	140

2.23.1 Purification of polyclonal antibodies

Polyclonal antibodies were purified using a Nunc ProPur^{TM} Midi G protein purification kit (Fisher Scientific, Loughborough, UK). Pre-packed resin Midi plug containing immobilised recombinant Protein G resin was loaded into the barrel of a Propur spin column using the insertion tool provided. The protein G spin column was then pre-equilibrated with 10 ml of binding buffer A, pH 7.4 by centrifuging at 500 × *g* for 3 min. Polyclonal antibodies were filtered through a single 0.2 µm syringe filter and diluted with binding buffer A at 1:1 ratio. The mixture was mixed by inverting the tube 3-4 times. The mixture was pipetted into the spin column and centrifuged at 150 × *g* for 30 min. The spin column was washed twice with 10 ml binding buffer A to remove unbound material by centrifuging at 500 × *g* for 3 min. The bound IgG was eluted with 10 ml elution buffer B2 added directly into a new centrifuge tube that had been added with 1.3 ml neutralisation buffer C to bring the pH of the sample to approximately 7.5. This step was done by centrifugation at 500 × *g* for 3 min. The tube was swirled to ensure through mixing of the final eluate with neutralisation buffer C. Sodium azide was added to a final concentration of 0.2 % (w/v) and stored at -20°C until used.

For regeneration of the protein G Midi plugs, plugs was washed with 10 ml elution buffer B2 by centrifuging the spin columns at 500 × *g* for 3 min. The plugs were then washed with 10 ml binding buffer A by centrifugation at 500 × *g* for 3 min. The plugs was then stored in binding buffer A at 2-8°C until further use. Antibodies were concentrated using an Amicon Ultra-4 centrifugal filter device (Millipore, Fisher Scientific, Loughborough, UK).

2.24 Production of *B. mandrillaris* monoclonal antibodies

The whole process involved four phases: immunisation, fusion, cloning and hybridoma expansion. Production of *B. mandrillaris* monoclonal antibodies began with immunisation of five Balb/c mice (UK 4241, UK4242, UK4243, UK4244 and UK4245) by Cambridge Research Biochemicals, Cleveland, UK. A pre-immunisation bled was taken prior to the immunisation schedule and ELISA was performed to make sure mice were negative for *B. mandrillaris*. All mice were immunised with 1×10^6 of formalin-treated *B. mandrillaris* as the immunogen for five times subcutaneously. The first immunisation was performed with Freund's complete adjuvant while for the subsequent immunisations immunogen was emulsified Freunds incomplete adjuvant. ELISA was performed at the CRB for determining *B. mandrillaris* antibody levels in immunised mice. Samples of sera from test bleed 3 were shipped to University of Leicester for further evaluation. The immunisation schedule is shown in Table 2.5.

Table 2.5 Immunisation schedule for production of *B. mandrillaris* monoclonal antibodies

Procedure	Day
Pre-immune bleed and immunisation 1	0
Immunisation 2	21
Test bleed 1	31
ELISA	35
Immunisation 3	42
Test bleed 2	52
ELISA	56
Immunisation 4	63
Test bleed 3	73
ELISA	77
Immunisation 5	84
Proposed terminal bleed	87

For phase 2 (fusion), spleen cells from hyper-immunise mouse was prepared and fused with P3X63Ag8.653 myelomas using polyethylene glycol by Cambridge Research Biochemicals (CRB). Viable hybridomas were selected and screened for antigen specific antibodies by ELISA performed at the CRB. Media from positive hybridomas were shipped to University of Leicester for evaluation. Three positive clones were selected for continued for subcloning. Wells with growing cells following cloning were screened for antibody secretion by ELISA (Section 2.28) and media samples from the subclones with the highest absorbance determined by microplate reader were shipped to University of Leicester for further analysis.

2.24.1 Isotyping monoclonal antibodies

Antibody heavy chain and light chain of monoclonal antibodies isotype determination was performed using a Hycult biotechnology mouse monoclonal antibody isotyping kit (Cambridge Bioscience, Cambridge, UK). The method relies on the capture of mouse antibodies by isotype-specific rat anti-mouse monoclonal antibodies arrayed on a dipstick. One ml of monoclonal antibody was added into each test tube containing the dipstick. A volume of 500 µl buffer was added to the tube and mixed gently. Rat anti-mouse κ conjugate was resuspended by shaking the bottle and 1 ml was added to the test tube containing the dipstick. The contents were mixed by gentle shaking and tube was left at room temperature overnight with the dipstick facing down. Two spots should be seen (one isotype and κ).

2.24.2 Purification of monoclonal antibodies

Monoclonal antibodies was filtered using 0.2 µm pore size membrane (VWR International Ltd.) to remove debris prior to purification using a Montage antibody purification kit with PROSEP-G medium. The PROSEP-G plug was first inserted into a

spin column with the top (recessed) end uppermost, using the insertion tool provided. The spin column containing the plug was then put into a 50 ml centrifuge tube and the plug was pre-equilibrated with 10 ml Binding Buffer A by centrifugation at 500 × *g* for 5 min. Filtered monoclonal antibodies was diluted 1:1 in Binding buffer A and transferred into the spin column and centrifuged at 100-150 × *g* for 20 min. The spin column was then washed to remove unbound material by adding 20 ml Binding Buffer A and centrifugation for 5 min at 500 × *g*. The bound IgG was then eluted for four times each with 2.5 ml of Elution Buffer B2 directly into separate centrifuge tubes with each containing 0.325 ml Neutralisation Buffer C to bring the sample to neutral pH. This elution step was done by centrifugation at 500 × *g* for 5 min. Purified antibodies were stored at -20°C.

2.25 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

The procedures were performed as described by Sambrook & Russell (2001), using a Mini-PROTEAN 3 cell (Bio-Rad, Hemel Hempsted, UK).

2.25.1 Preparation of gel

Prior to gel casting, glass plates were assembled, aligned and placed on a casting stand. A volume of 15 ml 12% (w/v) resolving gel was prepared (Sambrook & Russell, 2001) using the components listed in Table 2.6.

Table 2.6 Components for preparing 12% (w/v) resolving gels

Component	**Volume for two gels (ml)**
Nanopure water	4.9
30% (w/v) acrylamide mix Ultrapure protogel – National Diagnostics, UK	6.0
1.5M Tris (pH 8.8)	3.8
10% (w/v) SDS	0.15
10% (w/v) ammonium persulfate	0.15
TEMED (Bio-Rad, UK)	0.006

Once the ammonium persufate and TEMED were added, the mixture was vortexed and dispensed into the assembled cassettes using a disposable pipette. Immediately, the resolving gels were overlaid with nanopure water to prevent dehydration and left to solidify for approximately 45 min. Once the gels had harden, the nanopure water was discarded and excess was absorbed with filter paper. Stacking gel was then prepared using the components in Table 2.7. Following the addition of ammonium persulfate and TEMED, the solution was quickly vortexed and dispensed on top of the resolving gels. The comb was immediately inserted and gel was allowed to solidify for 1 hour. For used on the same day, the prepared gels were transferred into the electrophoresis tank containing half-filled of 1× Tris-glycine-SDS (TGS) buffer otherwise the gels were stored in the buffer at 4°C. This buffer was prepared by diluting a 10× TGS stock (National Diagnostics) in nanopure water.

Table 2.7 Components for preparing 5% (w/v) stacking gels

Component	Volume for two gels (ml)
Nanopure water	3.4
30% (w/v) acrylamide mix Ultrapure protogel – National Diagnostics, UK	0.83
1.0M Tris (pH 6.8)	0.63
10% (w/v) SDS	0.05
10% (w/v) ammonium persulfate	0.05
TEMED (Bio-Rad, UK)	0.005

2.25.2 Sample preparation

Nanopure water was heated to boiling in a beaker. While waiting, the protein solution was diluted in DPBS to produce final concentrations of 4, 40, 400 µg/ml. The samples were then added with 4× NuPAGE LDS (lithium dodecyl sulphate) sample buffer to produce a 1× final concentration. The lids of the samples tubes were punctured with a needle, in order to release the pressure generated during the heating process. The tubes were then placed in a floating rack and transferred to the boiling water for 5 min.

2.25.3 Electrophoresis

The prepared samples were then loaded into wells of the gels (Section 2.25.1) alongside a protein molecular weight marker mixture (Precision plus protein standard - all blue, Bio-Rad). The electrophoresis tank was filled with 1× TGS buffer and lid was placed on the tank in the correct orientation of the electrodes. The gels were run at a constant current of 40 mA until the dye present in the loading buffer reached the bottom. Once the electrophoresis was completed, gels were carefully removed from the cassette for used in Western blotting.

2.26 Western blotting

2.26.1 Protein transfer

After SDS-PAGE, a gel sandwich was prepared in a plastic blotting cassette. The sandwich consisted of a sponge pad, two layers of blotting filter paper, SDS-PAGE gel, Amersham nitrocellulose membrane (GE Healthcare Life Sciences, Little Chalfont, UK), two layers of blotting filter paper and a sponge pad, as described by Sambrook & Russel, 2001. The cassette, with the gel was placed on the negative (dark) side of a blotting cassette and nitrocellulose membrane on the positive (clear) side was then placed in a mini trans-blot tank (Bio-Rad), filled with ice-cold transfer buffer (5.9 g Tris, 2.9 g glycine, 200 ml methanol, 3 ml 10% (w/v) SDS, 800 ml nanopure water, pH 8.3) placed in an ice bucket. The transfer was performed for 1 hr at 250 mA. Once the blotting was completed, the nitrocellulose membrane was then blocked overnight with 5% (w/v) skimmed milk in DPBS at 4°C in big Petri dishes.

2.26.2 Immunodetection

The nitrocellulose membrane from section 2.26.1 was placed in a Petri dish and washed 3 times (10 min each) by soaking in DPBS containing 0.05% (v/v) Tween-20 (DPBS-T) on a shaker. The edges of the membrane were trimmed with a scapel and transferred to a new Petri dish. Following this, primary antibody diluted 1:1000, was prepared in DPBS-T plus 2% (w/v) skimmed milk. It was then added to the Petri dish containing the membrane and incubated for 90 min at RT on a shaker. The membrane then was washed four times, as mentioned above. The membrane was then incubated with secondary antibody, goat anti-mouse IgG conjugated with alkaline phosphatase (1:2000 dilution in DPBS-T plus 2% (w/v) skimmed milk), at room temperature and shaking for 1 hour. The membrane was washed again with DPBS-T for 3 times. The detection of the

antibody complex was done by incubating the membrane with approximately 5 ml of BCIP/NBT liquid substrate system until the bands were completely developed.

2.27 Coating of microtitre wells

To determine *B. mandrillaris* antibody levels in experimental animals or for screening of supernatant for monoclonal antibodies, 96-well flat bottom plate (Nunc MaxiSorp, Fisher Scientific, UK) were initially coated with 25 µg/100 µl of sonicated *B. mandrillaris* prepared in coating buffer (Appendix 1). Plates were then stored at 4°C overnight prior to ELISA assays.

For specificity testing, the 96-well plate was coated with suspension of free-living amoebae (*B. mandrillaris* (ATCC 50209), *A. castellanii* (ATCC 50370) and *N. gruberi* (ATCC 30224) prior to ELISA, immunoperoxidase and immunofluorescence assays. Briefly, a 100 µl suspension containing approximately 2×10^4 trophozoites or cysts in DPBS was added onto each 96 well plate and allowed to adhere for about 40-60 min. Excessive medium was subsequently removed by pipetting. The adherent amoebae were fixed with 100 µl of cold methanol for 30 min at -20°C. The methanol was then discarded and the plate was air-dried and stored at 4°C until used.

2.28 Enzyme-Linked Immunosorbent Assay (ELISA)

Amoebae or *B. mandrillaris* lysate were coated onto 96-well plate as described in Section 2.27. Plates were removed from 4°C and washed with 0.05% (v/v) Tween 20 in DPBS (0.05% (v/v) T-DPBS, (Appendix 1). The plates were blocked with 5% (w/v) skimmed milk in 0.05% (v/v) T-DPBS (Appendix 1) for 5 min and washed for three times with 0.05% (v/v) T-DPBS by pipetting. Dilutions of primary antibodies were prepared in 0.05% (v/v) T-DPBS + 1% (w/v) skimmed milk (Appendix 1) in separate 96-well plate and 100 µl added to each coated well and left at 37°C for 90 min. The

plate was washed three times with 0.05% (v/v) T-DPBS. A hundred µl of goat anti-rabbit IgG peroxidase conjugate (1:10,000) or goat anti-mouse IgG peroxidase conjugate (1:4000) prepared in 0.05% (v/v) T-DPBS + 1% (w/v) skimmed milk was added and incubated for another 1 hr at 37°C. Plate was washed three times with 0.05% (v/v) T-DPBS. Subsequently, 100 µl of tetramethyl benzidine (TMB) peroxidase enzyme immunoassay (EIA) substrate (Bio-Rad, Hemel Hempstead, UK), was added and left for 15 min at 37°C to allow colour development. The reaction was stopped with the addition of 0.18M sulphuric acid (Appendix 1). Results were assessed by visual inspection of the colour intensity compared to control well. In certain cases, the absorbance was measured at 450 nm.

Statistical analysis was performed by two-way analysis of variance (ANOVA) or t-test (Mann-Whitney), as appropriate. Bonferroni post-test analysis was performed when two-way ANOVA was used. Statistical significance was assumed at $P<0.05$.

2.29 Immunoperoxidase assay

Free-living amoebae were coated onto 96-well plate as described in Section 2.27. The plate was washed with 0.3% (v/v) Tween 20 in DPBS (0.3% (v/v) T-DPBS, Appendix 1). The plate was then blocked with 3% (w/v) skimmed milk in 0.3% (v/v) T-DBPS for 30 min and washed with 0.3% (v/v) T-DPBS for three times. Dilutions of 1:2500, 1:25,000 and 1:250,000 polyclonal antibodies (Section 2.23) were prepared in 3% (w/v) skimmed milk in 0.3% (v/v) T-DPBS. A volume of 100 µl of each dilution was added into each well of the 96-well plate, and the plate was covered and incubated at 37°C for 90 min. The plate was then rinsed three times with 0.3% (v/v) T-DPBS. Peroxidase-conjugated goat anti-rabbit IgG (Sigma-Aldrich) was diluted 1:10,000 in 3% (w/v) skimmed milk in 0.3% (v/v) T-DPBS and 100 µl was added into each well. The plate

was covered and further incubated at 37°C for 1 hr. The plate was rinsed three times with 0.3% (v/v) T-DPBS and 100 µl of TrueBlue peroxidase substrate (KPL, Insight Biotechnology, Wembley, UK) was added and left for 30 min. The reaction was stopped by rinsing the wells with nanopure water prior to viewing for blue staining of cells with an inverted microscope (CKX41, Olympus).

2.30 Immunofluorescence assay (IFA)

Plate of 96 wells was initially coated with free-living amoebae as described in Section 2.27. The plate was washed with 0.3% (v/v) Tween 20 in DPBS (0.3% T-DPBS, Appendix 1) and blocked with 3% (w/v) skimmed milk in 0.3% (v/v) T-DBPS for 30 min (Appendix 1). The plate was washed with 0.3% (v/v) T-DPBS for three times. Two-fold dilutions (1:50-1:25600) of polyclonal antibodies (Section 2.23) was prepared in 3% (w/v) skimmed milk in 0.3% (v/v) T-DPBS in a 96-well plate. A volume of 100 µl of each dilution was added into each well of the coated plate and the plate covered and incubated at 37°C for 90 min. The plate was then rinsed three times with 0.3% (v/v) T-DPBS. Goat anti-rabbit IgG FITC conjugate (Sigma-Aldrich) was diluted 1:80 in 3% (w/v) skimmed milk in 0.3% (v/v) T-DPBS and 100 µl was added into each well. The plate was covered and further incubated at 37 °C for 1 hr. The plate was rinsed three times with 0.3% (v/v) T-DPBS, mounted with 2.5% 1,4-diazobicyclo-[2.2.2]-octane (DABCO) mounting medium (Appendix 1) prior viewing with a fluorescence microscope (CKX41-URFLT50, Olympus). The intensity of fluorescence was scored from ± to 4+. Control wells were prepared by incubating the coated wells with secondary antibody only. Whereas, the secondary antibody for immunofluorescence assay using monoclonal antibodies was goat anti-mouse IgG FITC conjugate (Sigma-Aldrich) with a dilution of 1:70 prepared in 3% (w/v) skimmed milk in 0.3% (v/v) T-DPBS.

2.31 *B. mandrillaris* trophozoites for drug sensitivity testing

B. mandrillaris was initially cultured on MA104 monolayers, as described in Section 2.10. The *B. mandrillaris* was then harvested by centrifugation at 800 × *g* for 3 min and the pellet was transferred and maintained in BM3 axenic culture medium at 37°C (Schuster *et al.*, 1996). Briefly, a basal medium was prepared by adding 2 g of biosate peptone, 2 g of yeast extract and 0.5 g of torula yeast RNA to 345 ml of nanopure water and sterilised by autoclaving. Then, 34 ml 10× Hanks' balanced salts solution (HBSS), 100 ml 5% (w/v) neutralised liver digest in HBSS (Appendix 1), 5 ml 100× MEM vitamins mixture (Sigma-Aldrich, Dorset, UK) 0.5 ml 1000× lipid mixture (Sigma-Aldrich, Dorset, UK), 5 ml 100× MEM non-essential amino acids (Sigma-Aldrich, Dorset, UK), 5 ml 10% (w/v) glucose, 0.5 ml of hemin at 2 mg/ml and 5 ml of 0.5% (w/v) taurine (filter-sterilised) was aseptically added into the basal medium. If necessary, the pH was adjusted to 7.2 with 1M NaOH. Prior to use, 10% (v/v) heat-inactivated newborn calf serum (Sigma-Aldrich, Dorset, UK) was added to obtain a complete medium.

2.32 Drug sensitivity testing

The drugs tested are given in Table 2.8. The assay was performed in a 96-well flat bottom plate (Helena Biosciences, Gateshead, UK). Stock solutions (1000 µM) of drugs were prepared using appropriate solvents, except Amphotericin B which was purchased ready-made at 250 µg/ml. Two hundred µl of each drug was added to column 1 rows A-H of a 96-well plate. One hundred µl BM3 medium was added to the rest of the wells. The drug solutions from column 1 were then serially diluted two-fold in BM3 medium across the plate from column 1-11, to produce concentrations from 1000 µM to 1 µM.

Well 12 served as control well and contained no drug. The details of the assay for *B. mandrillaris* trophozoites and cysts are described in Sections 2.33 and 2.35.

2.33 Drug assay with *B. mandrillaris* trophozoites

B. mandrillaris trophozoites were adjusted to a final concentration 1×10^5/ml in BM3 medium. A volume of 100 µl of the *B. mandrillaris* trophozoites suspension was added into each well containing serially diluted drugs, prepared as in Section 2.32. The addition of this suspension diluted the initial drug concentrations of 1000 µM to 1 µM to final concentrations of 500 µM to 0.5 µM. Plates were incubated at 37°C for 48 hrs. Subsequently, the drug-containing medium was carefully removed using a pipette and 200 µl fresh BM3 medium was then added into each well. A sample from each well was transferred to a monolayer of MA104 grown in a 96-well plate and incubated at 37°C with 5% (v/v) CO_2 for 7 days. The plate was observed regularly, using an inverted microscope, for clearing of the monolayer and the presence of normal branching trophozoites. The remaining *B. mandrillaris* trophozoites in BM3 medium was re-incubated for up to seven days and any changes in the amoeba morphology was observed and recorded. The minimum trophozoite amoebacidal concentration (MTAC) was determined as the lowest concentration of drug resulted in complete lysis or degeneration of the trophozoites by seven days incubation.

Table 2.8 Drugs used in this study

Drug	Supplier	Solvent
Amphotericin B solution	Sigma Aldrich, Dorset, UK	Prepared in deionised water
Diminazene aceturate (Berenil®)	Sigma Aldrich, Dorset, UK	Nanopure water
Ciclopirox olamine	Sigma Aldrich, Dorset, UK	10% (v/v) methanol
Miltefosine	Sigma Aldrich, Dorset, UK	Nanopure water
Natamycin (Pimaricin)	Molekula Ltd, Dorset, UK	100% DMSO
Paromomycin sulphate	Sigma Aldrich, Dorset, UK	Nanopure water
Pentamidine isethionate	Sigma Aldrich, Dorset, UK	Nanopure water
Protriptyline hydrochloride	Sigma Aldrich, Dorset, UK	Nanopure water
Spiramycin	Sigma Aldrich, Dorset, UK	20% (v/v) ethanol
Sulconazole nitrate	Sigma Aldrich, Dorset, UK	Nanopure water
Telithromycin	Sanofi- Aventis, Surrey, UK	20% (v/v) ethanol

2.34 Preparation of *B. mandrillaris* cysts for drug sensitivity testing

B. mandrillaris cysts were produced once the monolayer that was used to feed the *B. mandrillaris* was destroyed (Section 2.10). The cysts were harvested on day-10 by centrifugation at 800 × *g* for 3 min and washed once with DPBS by centrifugation. The pellet was then resuspended in DPBS containing 0.3% (v/v) Sarkosyl and vortexed briefly to remove any remaining trophozoites and immature cysts. The cyst suspension was then washed twice with DPBS by centrifugation and cyst counts were performed using a modified Fuchs Rosenthal haemocytometer. Purified cysts were stored in DPBS at 4°C for use within 7 days. In addition, the encystment of *B. mandrillaris* trophozoites was also tested by incubating 2×10^5/ml of *B. mandrillaris* trophozoites in RPMI-L-glutamine medium containing 100 mM of galactose at 37°C for 48 hours (Siddiqui *et al.*, 2010). The presence of *B. mandrillaris* cysts was observed using an inverted microscope and cell counts were performed as mentioned above.

Other methods tested for the production of *B. mandrillaris* cysts were using *Acanthamoeba* encystment media or agent with Neff's (0.1 M potassium chloride, 0.008 M magnesium sulphate, 0.0004 M calcium chloride, 0.001 M sodium hydrogen carbonate and 0.02 M Tris buffer) (Neff *et al.*, 1964), NMT (86 mM NaCl, 15 mM $MgCl_2$ and 20 mM taurine) (Srivastava *et al.*, 1983) and epinephrine (5 mM) (Srivastava *et al.*, 1983). For this, 2×10^5/ml of *B. mandrillaris* trophozoites were resuspended in the appropriate medium and incubated at 32°C for up to 7 days. The presence of *B. mandrillaris* cysts were observed using an inverted microscope and cyst counts were performed as mentioned above.

2.35 Drug assay for *B. mandrillaris* cysts

The drug assay against *B. mandrillaris* cysts was based on the ability of the *B. mandrillaris* cysts to revert to the trophozoite stage (excyst) following 48 hours of drug exposure. Serial two-fold dilutions of drugs were prepared in a 96-well flat bottom plate. Cysts (Section 2.34) were adjusted to 1×10^5/ml in BM3 and 100 µl were added into each of the well. Plates were incubated at 37°C for 48 hrs. Following this, drug-containing medium were removed by pipetting and 200 µl of fresh BM3 medium added into each well. A sample of the suspension in each well was transferred onto a monolayer of MA104 and incubated at 37°C with 5% (v/v) CO_2. The remaining *B. mandrillaris* cysts suspension in BM3 medium was re-incubated at 37°C. A total of 15-20 *A. castellanii* trophozoites (ATCC 50370) were then added into each of the well on day-5 after addition of the fresh BM3 medium. Plates were re-incubated for another seven days giving a total of 12 days incubation, following addition of BM3 medium. Plates were observed regularly for the presence of *B. mandrillaris* trophozoites using an inverted microscope (CKX41, Olympus). The minimum cysticidal concentration (MCC) was defined as the lowest concentration of drug that resulted in no excystment after 12 days of incubation.

To determine the effect of organic solvents on *B. mandrillaris*, serial two-fold dilutions of the organic solvents used to dissolve the drugs were prepared in wells of a 96-well plate. The *B. mandrillaris* trophozoites and cysts were adjusted in BM3 to a final concentration of 1×10^5 cells/ml. Following that, 100 µl of the trophozoite or cyst suspensions were added into each well and plates were incubated for 48 hrs at 37°C. The organic solvent containing medium was carefully removed by pipetting and replaced with 200 µl of fresh BM3 medium. Plate with *B.mandrillaris* trophozoites was reincubated for another 7 days and any morphological changes were observed. For

cysts, each of the wells was inoculated with 15-20 *A. castellanii* trophozoites on day-5 after replacement with fresh BM3 medium and reincubated for up to 12 days to allow for excystment to occur and observation was done with inverted microscope (CKX41, Olympus)

2.36 Toxicity of drugs on mammalian cells (MA104)

MA104 monolayer was initially grown in a 96-well plate as described in Section 2.10. Two-fold dilutions of drugs were prepared in a separate 96-well plate. Following this, the medium used to grow the MA104 monolayer was removed and cells were rinsed once with DPBS. Fifty microlitre of fresh maintenance medium containing DMEM with 2mM L-glutamine was then added into each well. Fifty µl of each drug dilutions was then added into wells with MA104 monolayers. The plate was incubated at 37°C with 5% (v/v) CO_2 for 48 hrs.

Two methods were used to determine the toxic effects of the tested drugs. The first was a colorimetric method using a CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay (Promega, Southampton, UK). For this, to each well 5 µl of the CellTiter 96 solution was added and the plate re-incubated at 37°C for another 4 hrs. The absorbance at 490 nm was measured.

The toxicity also was determined by observation, at 200× and 400× magnification with an inverted microscope, of rounded or lysed cells and disintegration of the monolayer. The minimum cytotoxic concentration (MA104 toxicity) was defined as minimum concentration at which all MA104 cells were rounded, lysed or detached from the well's surface.

2.37 Isolation of *B. mandrillaris* using Dynabeads FlowComp Flexi kit

The kit from Invitrogen, Paisley, UK was made of parts A and B. Part A contained chemicals used for isolation of cells using paramagnetic beads coated with streptavidin. Part B contained chemicals, including 'DSB-X' Biotin needed for labelling antibodies prior to cell isolation. Initial optimisation with pure *B. mandrillaris* trophozoites (ATCC 50209) was done using the suggested isolation buffer, containing Dulbecco A phosphate buffered saline (DPBS), 0.1% (w/v) bovine serum albumin (BSA) and 2 mM EDTA and the manufacturer's protocol.

2.37.1 Biotinylation of polyclonal antibodies

Purified polyclonal antibodies were diluted in sterile DPBS (Appendix 1) to give a final concentration of approximately 4 mg/ml. One millilitre of diluted polyclonal antibodies was transferred to a reaction tube containing a stir bar (Component C) and 100 µl of a freshly prepared 1 M sodium bicarbonate solution added. Forty microlitres of DMSO (Component B) was then added to one vial of 'DSB-X' biotin succinimidyl ester (Component A) and the contents dissolved by pipetting. While stirring, 30 µl of reactive DSB-X biotin solution was added to the reaction tube containing the antibody and 1M sodium bicarbonate and mixed thoroughly. The reaction mixture was left to stir for 90 min at room temperature. A piece of dialysis tubing (Component G) was rinsed in distilled water (dH_2O). A knot was tied in one end of the dialysis tubing and the excess dH_2O was squeezed out. The entire reaction mixture was then transferred to the tubing. A knot was tied in the other end of the dialysis tubing, leaving as little space between the knots as possible to prevent further dilution of the antibody. The dialysis tubing was hung in a 500 ml bottle containing a stir bar and filled with DPBS. The reaction mixture was dialyzed at 2-8°C for 24 hours with three changes of buffer. During the dialysis, the

buffer was gently stirred. The labelled antibody was stored in aliquots at -20°C until used.

2.37.2 Modified method for the isolation of *B. mandrillaris* trophozoites Dynabeads FlowComp Flexi kit

A total of 1×10^5 *B. mandrillaris* trophozoites were resuspended in 500 µl BM3 medium and added with 25 µl of DSB-X labelled polyclonal antibody (Section 2.37.1). The suspension was mixed by pipetting and incubated for 10 min at 4°C. The cells was added with 2 ml of cold BM3 medium and washed by centrifugation for 8 min at 400 × *g*. The supernatant was then discarded and cell pellet was resuspended with 1 ml of BM3 medium. Following this 75 µl of washed FlowComp Dynabeads (prepared by 2× centrifugation in BM3) was added and mixed well by pipetting. The suspension was incubated for 15 min at 4°C under rolling and tilting. The tube was then placed in the magnet for a minimum 1 min. The supernatant was carefully removed and discarded. The tube was removed from the magnet. A volume of 1 ml of cold BM3 medium was added and the bead-bound cells were resuspended by gentle pipetting for five times. The tube was placed again in the magnet for a minimum of 1 min and the supernatant was removed carefully by pipetting. The tube was removed from the magnet and carefully the bead-bound cells were resuspended in 2 ml of BM3 medium and centrifuged for 8 min at 400 × *g*. The supernatant was discarded and the cell pellet was resuspended in 10 ml of BM3 medium. The suspension was transferred into a culture flask and incubated at 37°C. The flask was checked for any morphological changes to the *B. mandrillaris* trophozoites, using an inverted microscopy.

2.37.3 Modified method for the isolation of *B. mandrillaris* cysts using Dynabeads FlowComp Flexi kit

A total of 1×10^5 *B. mandrillaris* cysts were resuspended in 500 µl Isolation buffer 1(IB1) containing DPBS and 0.1% (w/v) BSA and added with 25 µl of DSB-X labelled polyclonal antibody (Section 2.37.1). The suspension was mixed by pipetting and incubated for 10 min at 4°C. The cells was added with 2 ml of cold IB1 buffer and washed by centrifugation for 8 min at 400 × *g*. The supernatant was then discarded and cell pellet was resuspended with 1 ml of IB1 buffer. Following this 75 µl of FlowComp Dynabeads was added and mixed well by pipetting. The suspension was incubated for 15 min at 4°C with rolling and tilting. Tube was then placed in the magnet for minimum 1 min. Supernatant was carefully removed and the tube was removed from the magnet. A volume of 1 ml of cold IB1 buffer was added and the bead-bound cells were resuspended by gentle pipetting for 5 times. The tube was placed again in the magnet for a minimum of 1 min and the supernatant was removed carefully by pipetting. The tube was removed from the magnet and carefully the bead-bound cells were resuspended in 1 ml FlowComp Release Buffer. The tube was incubated for 2 min at room temperature under rolling and tilting. The suspension was mixed five times by pipetting and the tube was placed in the magnet for 1 min. The supernatant was transferred into a new tube and 2 ml of IB1 buffer was added. The tube was centrifuged for 8 min at 400 × *g* and supernatant was discarded. The cell pellet was resuspended in 10 ml of BM3 medium and the suspension was transferred into a culture flask and incubated at 37°C. After 24 hrs, a sample was transferred onto a flask containing a MA104 monolayer to determine the viability of the isolated cysts. The flask was checked for the presence of normal branching trophozoites and clearing of the monolayer, using an inverted microscopy.

3 Culturing *B. mandrillaris* on different cell lines, free-living amoebae, bacteria and fungus

3.1 Introduction

Little is known about *B. mandrillaris* growth requirements although studies have shown that the amoeba feed on other free-living amoebae, including *Acanthamoeba* and *Naegleria* but not on bacteria (Dunnebacke *et al.*, 2004, Visvesvara *et al.*, 1993, Matin *et al.*, 2006a). Because of this limitation, the amoeba is hard to be isolate using the conventional non-nutrient agar plates seeded with *E. coli* that is commonly used for isolation of other free-living amoebae (Schuster, 2002).

Besides feeding on other free-living amoebae, *B. mandrillaris* has been shown to feed on mammalian cells, including primary human brain microvascular endothelial cells and monkey kidney cells (Matin *et al.*, 2006a, Visvesvara *et al.*, 1993). However, very limited studies are available on the most appropriate type of mammalian cells that can be used for culturing *B. mandrillaris* in large numbers (Matin *et al.*, 2006a). This chapter describes experiments in which *B. mandrillaris* was cultured on different cell lines to investigate the most suitable mammalian cells that can be used in the laboratory for growing the amoeba in large-scale. In addition, the amoeba's preference for food sources when cultured with *Acanthamoeba*, *Naegleria*, bacteria and a fungus was also compared.

3.2 Results

3.2.1 Culture of *B. mandrillaris* on different cell lines

Three cell lines (MA104, Hep-2 and EA.hy926) were used to determine the most preferable food source for growing *B. mandrillaris*. For this, each cell line were initially grown into monolayers prior to the addition of *B. mandrillaris* trophozoites and incubated at 37 °C with 5% (v/v) CO_2. The results are summarised in Table 3.1. During the first 24 hrs of incubation, no clearing of monolayer was seen within any of the cell lines. The EA.hy926 monolayer was the fastest to be cleared by *B. mandrillaris*, and by 48 hrs incubation, almost 75% of the monolayer had been cleared, leaving only small patches of intact cells (Figure 3.1). At this time, *B. mandrillaris* trophozoites were seen all over the flask, with some still actively dividing (Figure 3.2). All the EA.hy926 cells were totally cleared by the trophozoites by 72 hrs.

For MA104 cell line, the monolayer was still intact at 48 hrs of incubation, although some cells had been damaged, as shown by the rounded morphology (Figure 3.3). Clearance of 50% of the monolayer could be seen as small gaps, after 72 hrs incubation (Figure 3.4). This clearance reached 75% by 96 hrs, with the remaining cell patches being completely cleared up by 120 hrs of incubation. Clear vesicles or small holes which were suspected to have been made by the tip of *B. mandrillaris* pseudopodia (Dunnebacke, 2007) were also seen on the MA104 monolayer (Figure 3.5). Figure 3.6 shows that almost the entire *B. mandrillaris* trophozoite had moved within a MA104 cell.

B. mandrillaris took the longest time to clear the tightly packed Hep-2 cells. After 48 hrs of incubation, high numbers of *B. mandrillaris* could only be seen in an area where the monolayer had accidently been torn by a pipette during a washing step (Figure 3.7).

Small gaps, indicating about 25% clearance of the monolayer, started to occur after 72 hrs (Figure 3.8) of incubation and reaching 75% by 120 hrs. *B. mandrillaris* trophozoites were seen occupying these cleared areas. Almost all the Hep-2 cells were cleared after 144 hrs. *B. mandrillaris* cell count was performed when each of the monolayers had been cleared by the amoeba. Highest *B. mandrillaris* cells count were obtained from flask with MA104 (1.2-1.7 × 10^5 cells/ml) followed by Hep-2 (5.3-6 × 10^4 cells/ml) and EA.hy926 (4.5-5 × 10^4 cells/ml).

Table 3.1 Degree of cell monolayer clearance by *B. mandrillaris*

Cell line	**Time of incubation (hrs)**					
	24	**48**	**72**	**96**	**120**	**144**
EA.hy926	-	++	+++	ND	ND	ND
MA104	-	-	+	++	+++	ND
Hep-2	-	-	±	+	++	+++

Results are of three independent experiments performed in triplicates

Degrees of clearance:

- = none

± = 25%

+ = 50%

++ = 75%

+++=100%

ND = not done

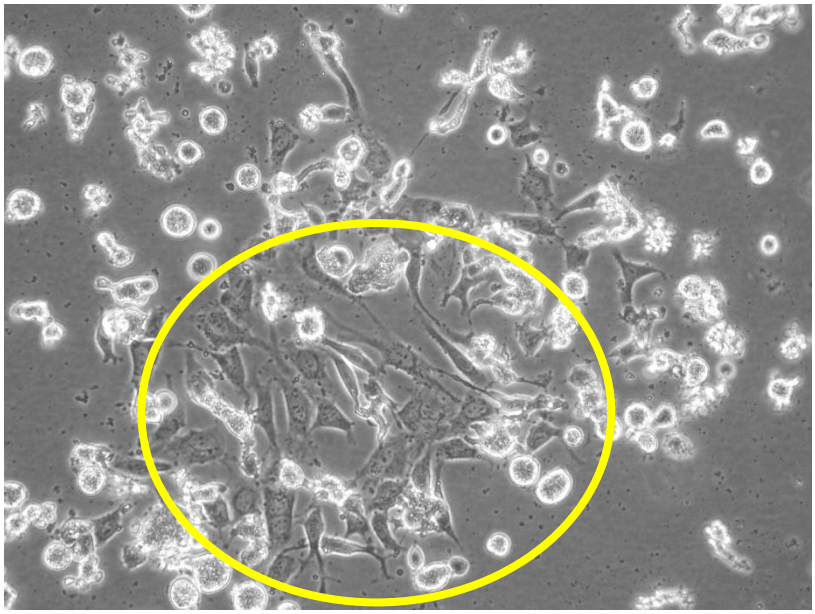


Figure 3.1 Monolayer of EA.hy926 after 48 hrs of incubation with *B. mandrillaris* trophozoites. Area in circle shows a patch of monolayer that had not be cleared by the amoeba (200×)

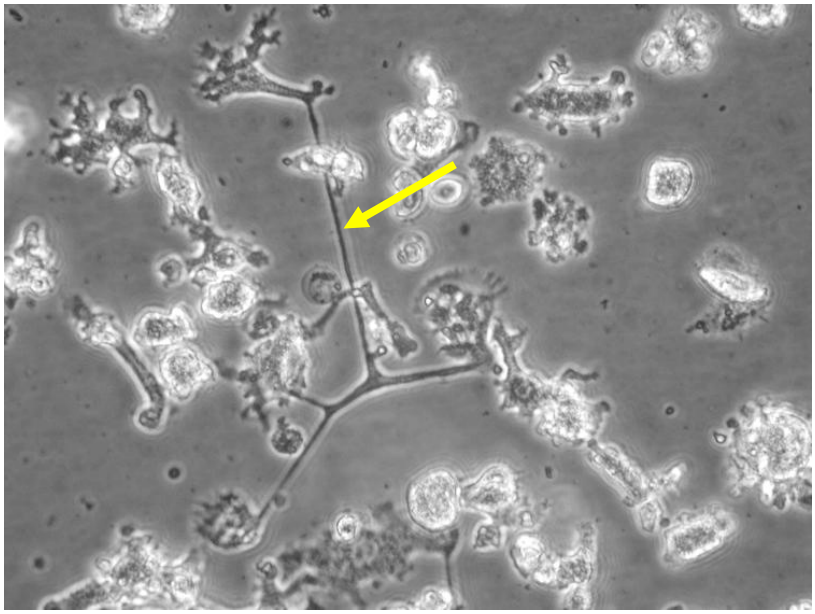


Figure 3.2 An actively dividing *B. mandrillaris* trophozoite (arrow) seen during growth with EAhy.926 monolayer (400×)

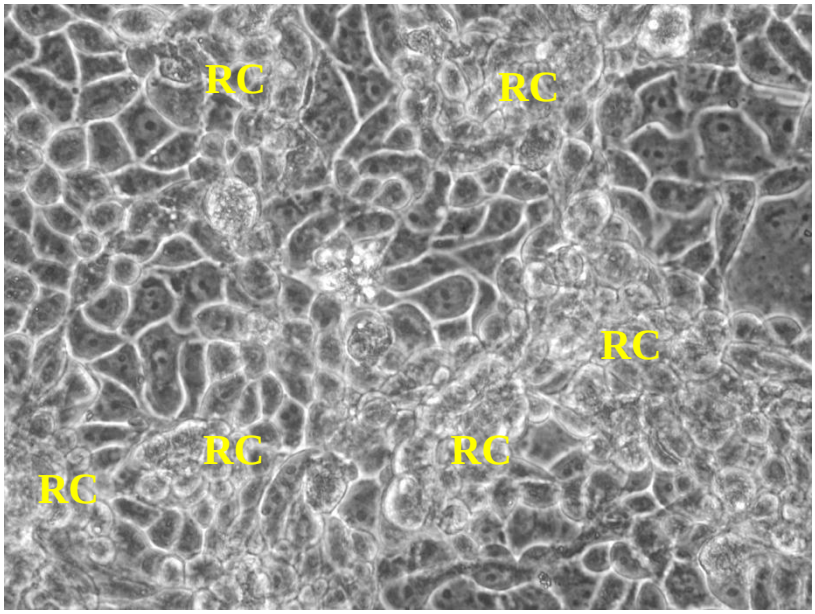


Figure 3.3 Damaged MA104 cells showing rounded morphology (**RC**) after 48 hrs incubation with *B. mandrillaris* (400×)

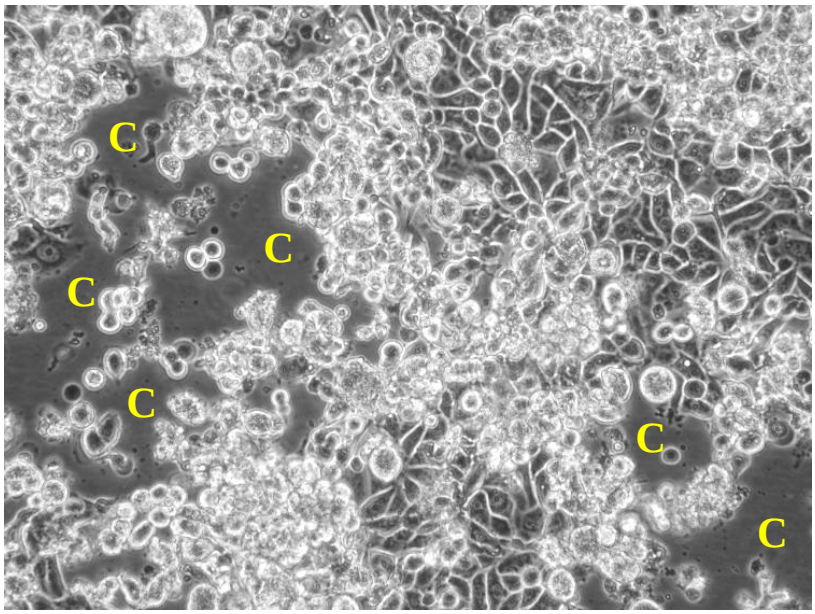


Figure 3.4 Monolayer of MA104 incubated with *B. mandrillaris* showing area of clearance (**C**) after 72 hrs of incubation (200×)

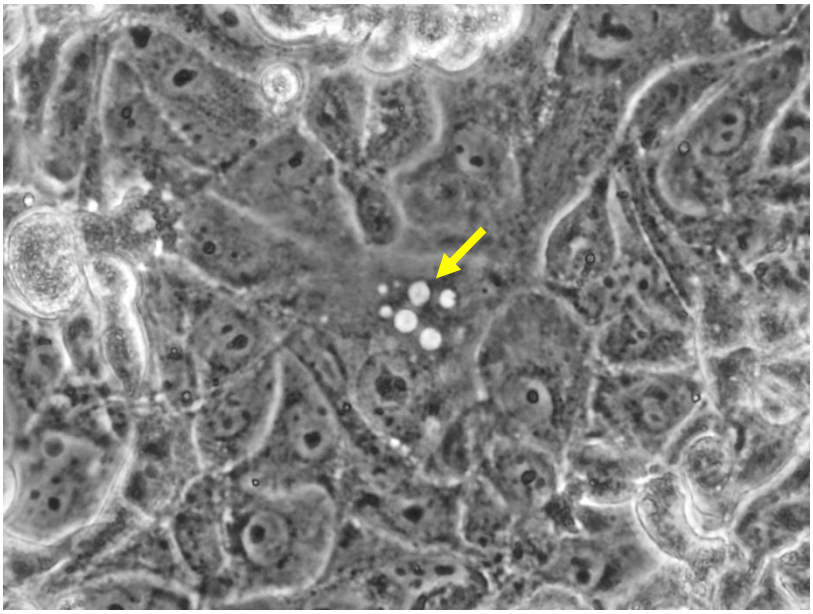


Figure 3.5 Clear vesicles or small holes that was suspected due to the tip of *B. mandrillaris* pseudopodia (Dunnebacke, 2007) seen on MA104 monolayer (400×)

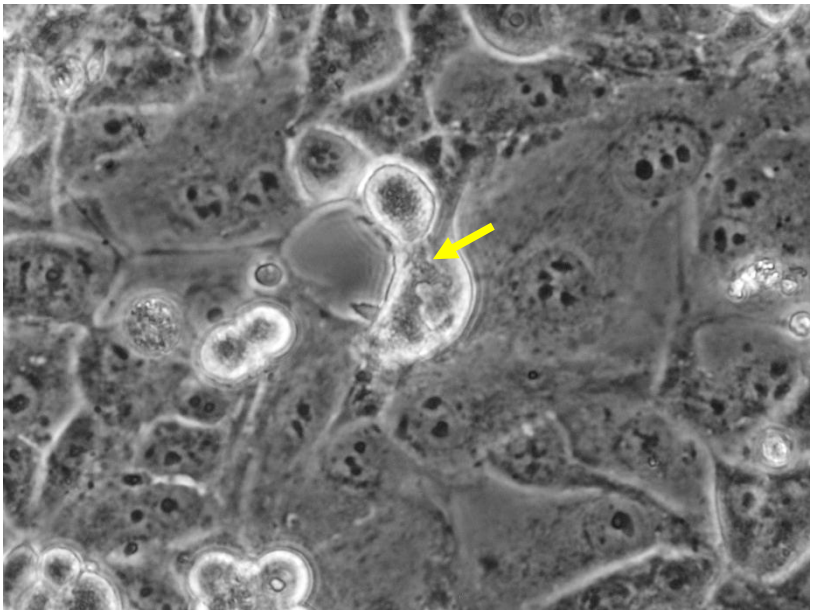


Figure 3.6 Almost the entire *B. mandrillaris* trophozoite (arrow) is within a MA104 cell (400×)

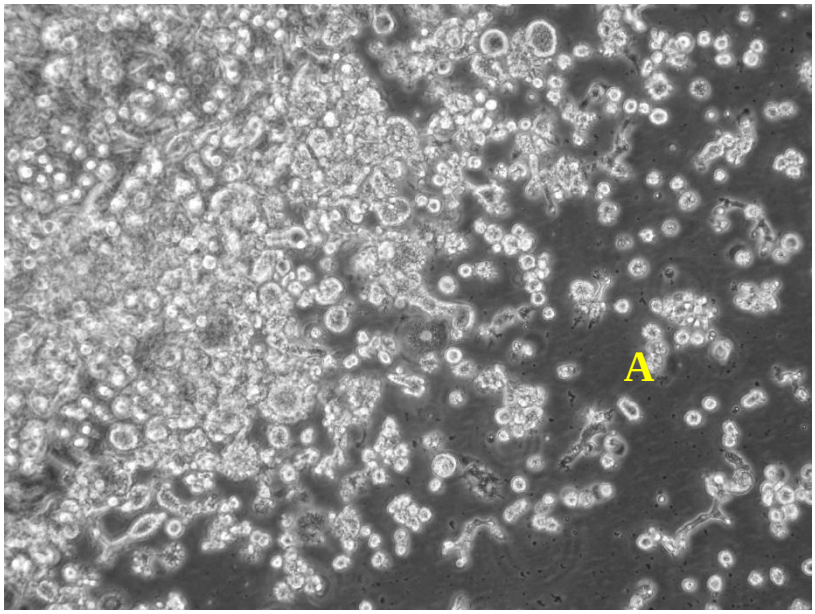


Figure 3.7 Abundant *B. mandrillaris* trophozoites seen in area (**A**) of Hep-2 monolayer which had accidently been removed during a washing step (200×)

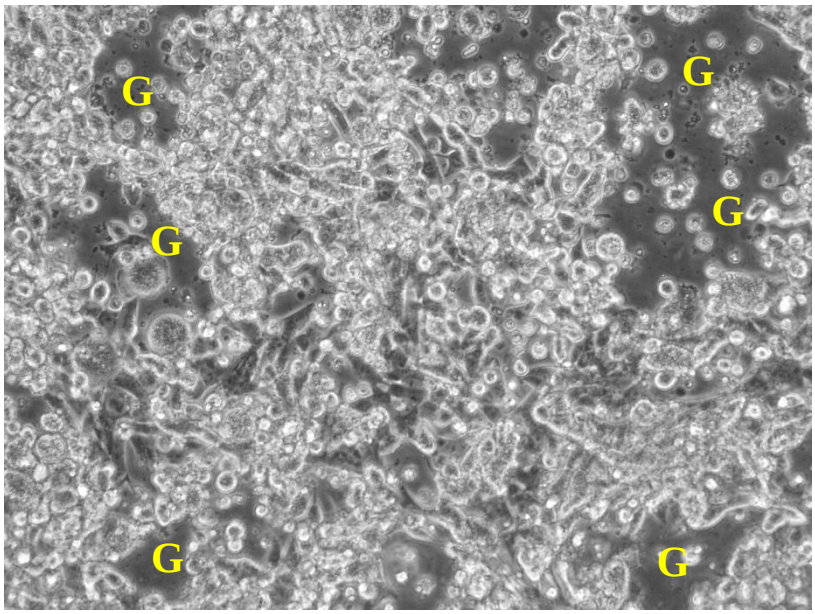


Figure 3.8 Tightly packed Hep-2 cells showing few gaps (G) after 72 hrs of incubation with *B. mandrillaris* trophozoites (200×)

3.2.2 Culture of *B. mandrillaris* with free-living amoebae, bacteria and a fungus

The activity of *B. mandrillaris* when fed with different free-living amoebae was evaluated based on the distance (cm) *B. mandrillaris* migrated on non-nutrient agar (NNA) and non-nutrient agar seeded with *Escherichia coli* (NNA-*E. coli*), lawned either with *Acanthamoeba castellanii* (ATCC 50370) trophozoites or *Naegleria gruberi* (ATCC 30224) trophozoites, or a combination of *A. castellanii* and *N. gruberi* trophozoites. In addition, the distance of migration was also determined on NNA plates lawned with bacteria *Bacillus* sp. (JPN), *Staphylococcus aureus* (ATCC 6538), *Stenotrophomonas maltophilia* (ATCC 15099) or *E. coli* (JM101). NNA plates lawned with the fungus *Candida albicans* (ATCC 10231) also were tested.

3.2.2.1 Culture of *B. mandrillaris* on NNA plates lawned with free-living amoebae

Table 3.1 shows the migration of *B. mandrillaris* trophozoites on NNA lawned with different types of free-living amoebae. On the NNA control plate, the *B. mandrillaris* trophozoites moved less than 1cm from the starting spot, even after 12 days of incubation. The *B. mandrillaris* trophozoites were smaller in size with less branching (Figure 3.9). Most of the *B. mandrillaris* trophozoites had changed into the dormant cyst stage by the end of the experiment.

When *B. mandrillaris* was incubated on NNA seeded with *A. castellanii* trophozoites, migration was 1 cm by day-3. Migration increased gradually from day-6 until day-9, reaching a maximum distance of 5 cm from the starting spot on day-12. Figure 3.10 shows *B. mandrillaris* trophozoites moved towards areas with high density of *A. castellanii* trophozoites. The *B. mandrillaris* trophozoites were seen to be more branching and elongated (Figure 3.11) than those seen on the control plate (Figure 3.9). Most of the *B. mandrillaris* was still in the trophozoites stage on day-12 but all the *Acanthamoeba* had encysted.

On NNA seeded with *N. gruberi* trophozoites, migration of *B. mandrillaris* was slow for the first 6 days of incubation, trophozoites moving less than 1 cm in this time. On day-9, most of the trophozoites were seen at area of 1-2 cm from the starting spot. A few *B. mandrillaris* trophozoites could be seen migrated up to 3 cm from the starting spot on day-12. Most of the *B. mandrillaris* was still in the trophozoite stage although some had encysted by 12 days of incubation. In contrast, almost all *N. gruberi* were dead at the end of the 12-day experiment.

On NNA plates seeded with equal numbers of *A. castellanii* and *N. gruberi*, the distance of *B. mandrillaris* migration on day-3 and day-6 was 1cm and 3-4 cm, respectively. Figure 3.12 shows *B. mandrillaris* trophozoites approaching *Acanthamoeba* and *Naegleria* trophozoites. By day-9, *B. mandrillaris* trophozoites were seen all over the plates. All *A. castellanii* had encysted and almost all *Naegleria* had died by day-12. Overall, *B. mandrillaris* showed more preference for *A. castellanii* trophozoites rather than *N. gruberi* trophozoites as the food source.

3.2.2.2 Culture of *B. mandrillaris* on NNA-*E. coli* plates lawned with free-living amoebae

Table 3.3 shows the distance of *B. mandrillaris* migration on NNA-*E. coli* plates lawned with different free-living amoebae. *B. mandrillaris* on NNA-*E. coli* control plate showed less than 1 cm migration by day-6. The maximum distance on this control plate on day-12 was 1.5 cm. The *B. mandrillaris* trophozoites were small in size.

On NNA-*E. coli* plates lawned with *A. castellanii* trophozoites, the distance the *B. mandrillaris* trophozoites travelled increased gradually from day-6 until day-9, reaching a maximum distance of 4 cm on day-12. The *B. mandrillaris* trophozoites were less elongated with thick branches (Figure 3.13). By day-12, most *Acanthamoeba* had

encysted (Figure 3.13) and very few *Acanthamoeba* trophozoites could be seen 6 cm from the starting spot.

For plates lawned with *E.coli* and *Naegleria* trophozoites, the maximum distance *B. mandrillaris* travelled was 2 cm and the appearance of the trophozoites were elongated with some showing many branches (Figure 3.14). When both *Acanthamoeba* and *Naegleria* were added onto NNA-*E.coli* plates, the majority of *B. mandrillaris* trophozoites were seen at area of 2-3 cm on day-9, reaching a maximum distance of 4 cm from the starting spot on day-12. By day-12, most *Acanthamoeba* had encysted and few *Acanthamoeba* and *Naegleria* trophozoites could be seen at area ≥ 5 cm from the starting spot. Overall, *B. mandrillaris* showed more preference for *A. castellanii* trophozoites rather than *N. gruberi* trophozoites as the food source.

Table 3.2 Distance of *B. mandrillaris* migration on NNA plates lawned with free-living amoebae

Amoeba	**Distance of *B. mandrillaris* migration (cm)**			
	Day-3	**Day-6**	**Day-9**	**Day-12**
None	<1	<1	<1	<1
A. castellanii	1	2-3	3-4	4-5
N. gruberi	<1	<1	1-2	2-3
A. castellanii + N. gruberi	1	3-4	>5	>5

Results are of three independent experiments performed in triplicates

Table 3.3 Distance of *B. mandrillaris* migration on NNA-*E. coli* plates lawned with free-living amoebae

Amoeba	**Distance of *B. mandrillaris* migration (cm)**			
	Day-3	**Day-6**	**Day-9**	**Day-12**
None	≤1	≤1	1-1.5	1-1.5
A. castellanii	1	1-2	2-3	3-4
N. gruberi	<1	<1	1-<2	1-2
A. castellanii + N. gruberi	1	1-2	2-3	3-4

Results are of three independent experiments performed in triplicates

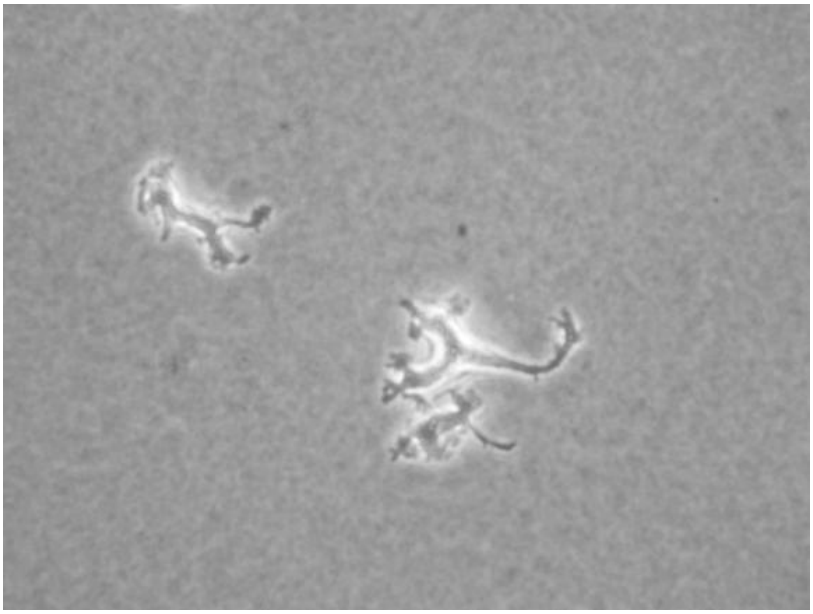


Figure 3.9 *B. mandrillaris* trophozoites on NNA without an amoeba lawn (control plate) (400×)

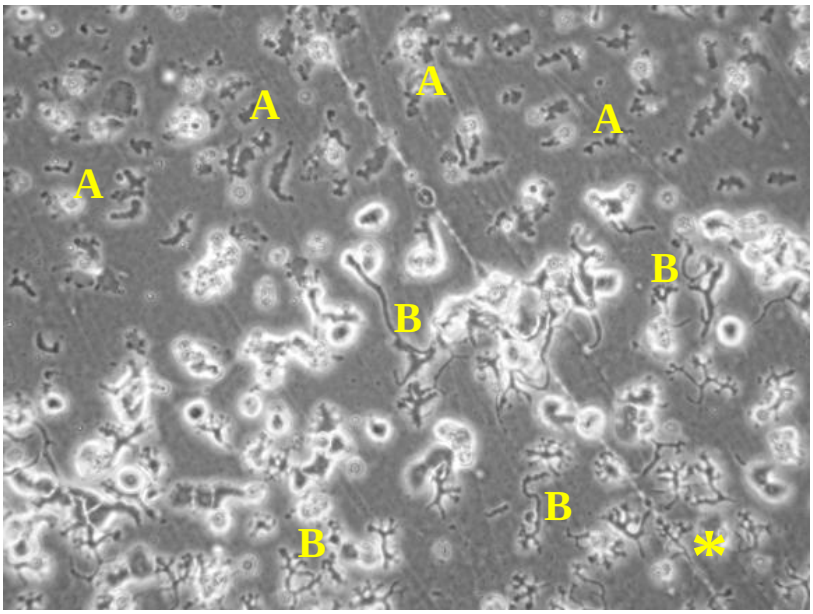


Figure 3.10 *B. mandrillaris* trophozoites (**B**) showed migration to areas with high numbers of *A. castellanii* trophozoites (**A**) following clearance of the amoeba in area (***)** near to the starting point **(**100×)

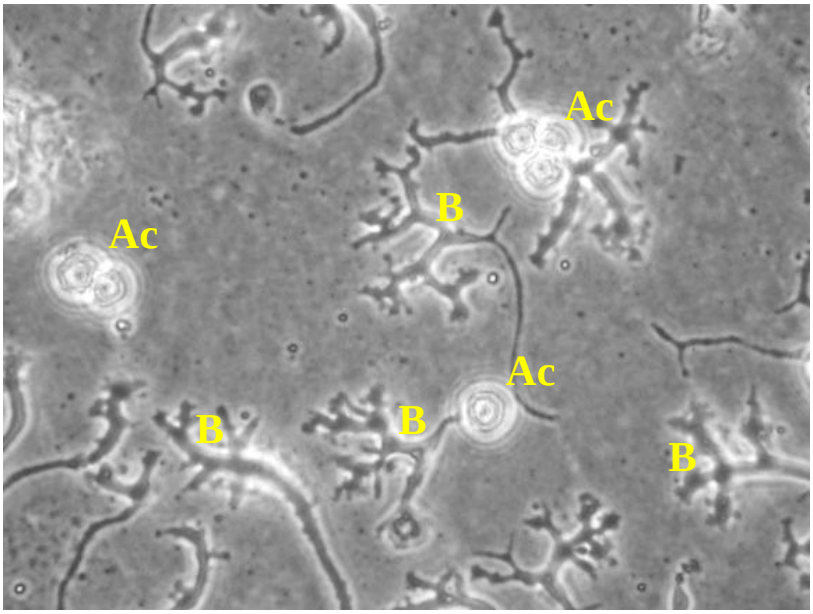


Figure 3.11 *B. mandrillaris* trophozoites (**B**) on NNA plate lawned with *A. castellanii* trophozoites showing elongated and branching morphology. **Ac** = *A. castellanii* cysts (400×)

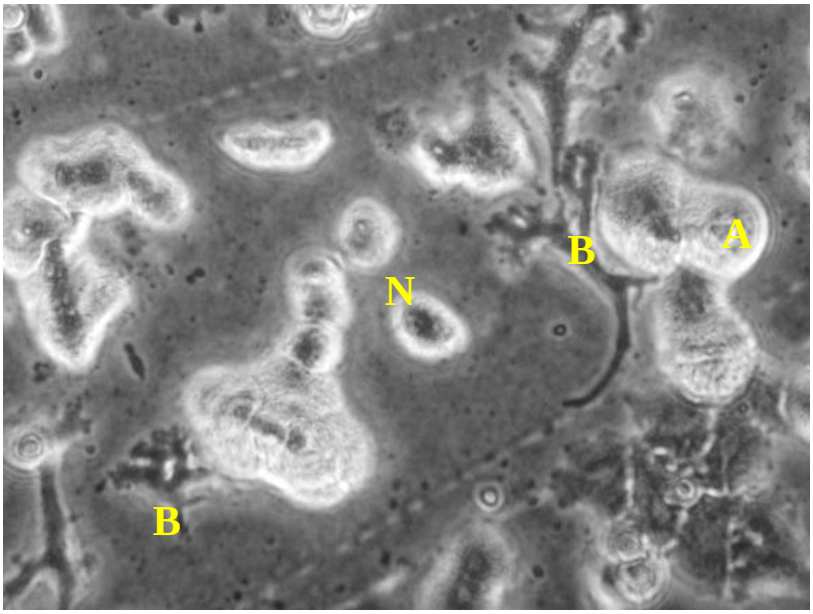


Figure 3.12 *B. mandrillaris* trophozoites **(B)** on NNA plate lawned with *A. castellanii* (**A**) and *N. gruberi* (**N**) trophozoites (400×)

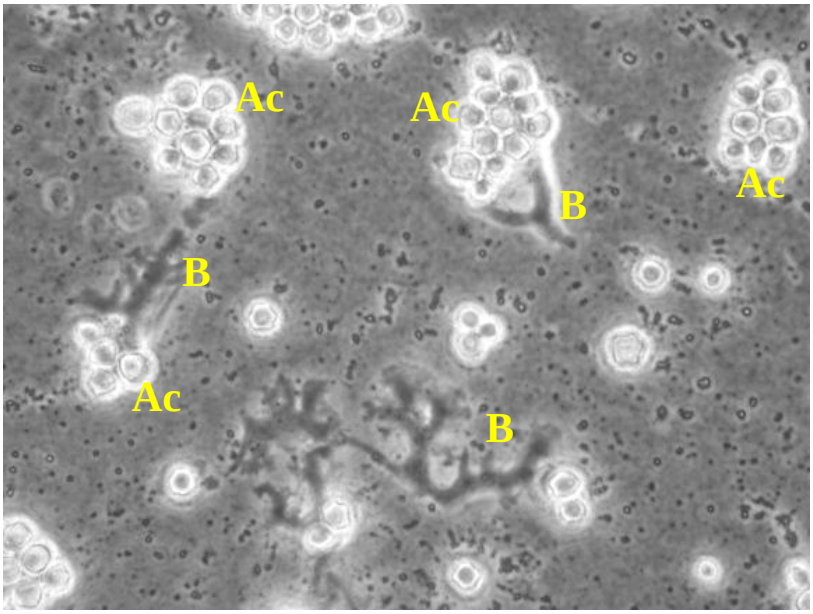


Figure 3.13 NNA-*E.coli* plate lawned with *A. castellanii* trophozoites incubated with *B. mandrillaris* trophozoites (**B**) as observed on day-12 showing high numbers of *A. castellanii* cysts (**Ac,** 400×)

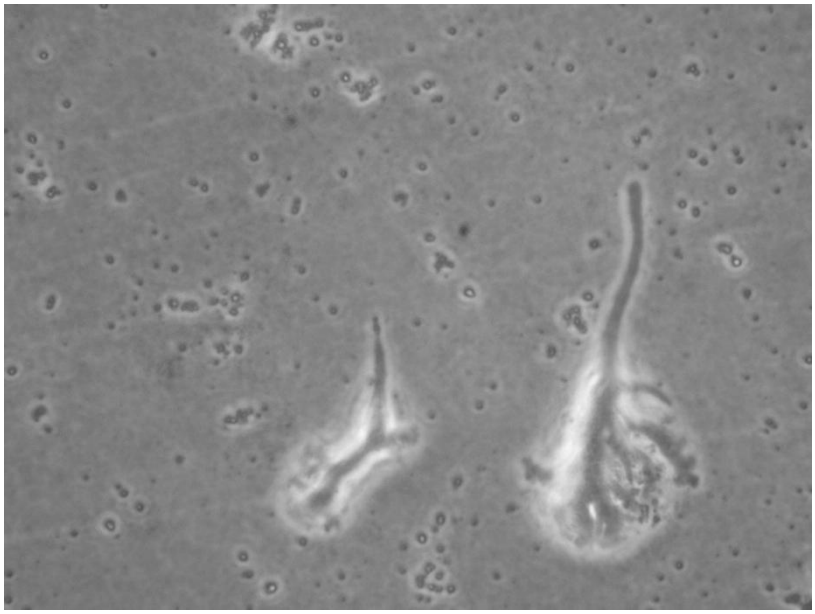


Figure 3.14 Morphology of *B. mandrillaris* trophozoites on NNA-*E. coli* plate lawned with *N. gruberi* (400×)

3.2.2.3 Culture of *B. mandrillaris* on NNA plates lawned with bacteria and a fungus

The distance of *B. mandrillaris* migration on NNA plates lawned with different types of bacteria or a fungus is summarised in Table 3.4. All plates showed less than 1 cm *B. mandrillaris* migration over 6 days of incubation. NNA plates lawned with *S. maltophilia*, S. *aureus* and *C. albicans* showed *B. mandrillaris* trophozoite migration up to 1 cm on day-12. The maximum distance that *B. mandrillaris* migrated by 12 days incubation was 1.5 cm and were observed on NNA plates lawned with *E. coli* or *Bacillus* sp. The *B. mandrillaris* trophozoites on both plates appeared thin and elongated (Figure 3.15 and Figure 3.16) in contrast to those observed on NNA-*E. coli* plates lawned with *Acanthamoeba* (Figure 3.13)

Table 3.4 Distance of *B. mandrillaris* migration on NNA plates lawned with bacteria and fungus

Bacteria and fungus	**Distance of *B. mandrillaris* migration (cm)**			
	Day-3	**Day-6**	**Day-9**	**Day-12**
None	<1	<1	<1	<1
E. coli	≤1	≤1	1-1.5	1-1.5
S. maltophilia	≤1	<1	<1-1	<1-1
S. aureus	≤1	≤1	≤1-1	≤1-1
Bacillus sp.	≤1	≤1	1-1.5	1-1.5
C. albicans	≤1	≤1	≤1-1	≤1-1

Results are of three independent experiments performed in triplicates

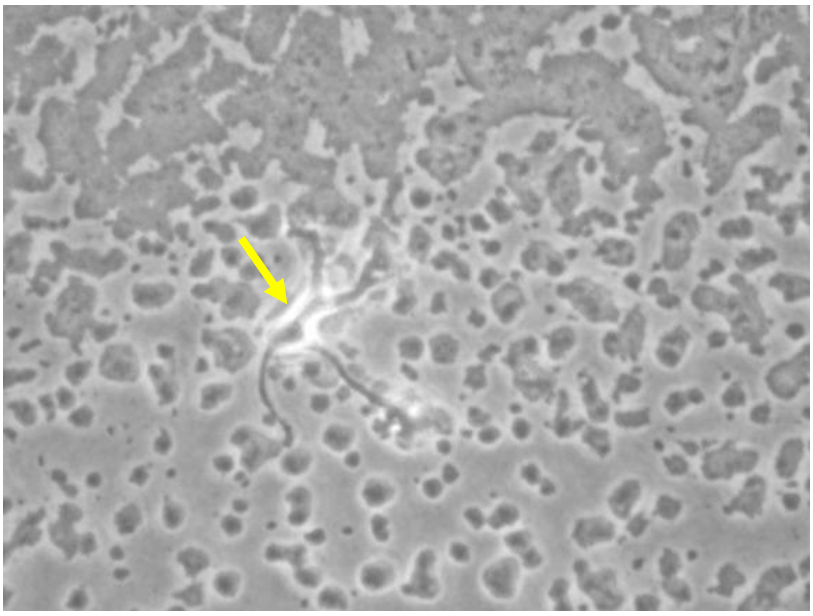


Figure 3.15 *B. mandrillaris* trophozoite (arrow) on NNA plate lawned with *Bacillus* sp. (400×)

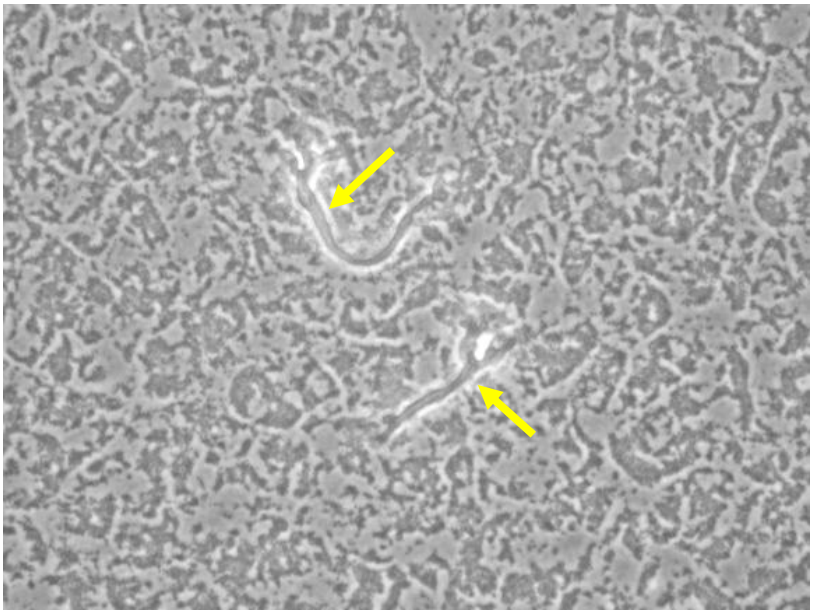


Figure 3.16 *B. mandrillaris* trophozoite (arrow) on NNA plate lawned with *E. coli* (400×)

3.3 Discussion

The ability to culture and produce large numbers of *B. mandrillaris* was a main pre-requisite for subsequent studies done in this thesis. Recently, good *in vitro* growth of *B. mandrillaris* has been shown using primary human brain microvascular endothelial cells (HBMEC) in contrast to monkey kidney fibroblast-like cells (COS-7) (Matin *et al.*, 2006a). In that study, an 8-9 fold increment of *B. mandrillaris* was seen using the HBMEC while those grown on COS-7 showed only 2-3 fold of increment (Matin *et al.*, 2006a). Unfortunately the HBMEC was not available commercially as immortal cells and a disadvantage of using primary endothelial cells is that they have a short live span and therefore are not suitable for long-term experiment (Bouis *et al.*, 2001). As an alternative, immortal EA.hy926 endothelial cells, derived from human umbilical vein, were tested. In addition, two other cell lines, African monkey kidney cells (MA104) and human cervix carcinoma cells (Hep-2), which were available in the laboratory, were also tested in parallel. The results showed that the highest *B. mandrillaris* cell count was obtained using the MA104 cell line, which was in contrast to what was predicted. It was expected that high numbers of *B. mandrillaris* would be obtained using the EA.hy926 cell line because it was an endothelial cell. The explanation for the contrast between the HBMEC and EA.hy926 endothelial cells could be that EA.hy926 has a different origin than the primary HBMEC. Studies have shown that endothelial cells that are derived from different organs or tissues vary in their morphology, growth characteristics and proteins expression which could cause the variations in the experimental results seen here (Craig *et al.*, 1998, Nylund *et al.*, 2010). For example, in a study involving endothelial cells that were derived from brain (THBMEC) and umbilical vein (HUVEC), a permanent expression of intercellular tight junction-proteins (e.g. ZO-1 and occludin) was observed with the THBMEC, while the HUVEC only showed

punctate expression of ZO-1 and absence of occludin (Man *et al.*, 2008). Peripheral blood mononuclear cell transmigration was also reported to be higher with HUVEC than the THBMEC (Man *et al.*, 2008). In addition, although both are endothelial cells, the EA.hy926 is macrovascular while HBMEC is a microvascular cell (Alsam *et al.*, 2003, Bouis *et al.*, 2001). Therefore, it is possible that this difference might also be one of the reasons for the contrast of *B. mandrillaris* counts obtained with the EA.hy926 cell line used in this study as compared to those with the HBMEC used by Matin *et al*. (2006a). In an angiogenesis study using microvascular cells from neonatal foreskin (FSE) and macrovascular endothelial cells from umbilical vein (HUVE), higher secretion of matrix metalloproteinase (MMP) and tissue inhibitor of MMP (TIMP1) was seen with the HUVE compared to the FSE (Jackson *et al.*, 1997).

In terms of preferable food source, the EA.hy926 was the fastest to be consumed (3 days) by the *B. mandrillaris* compared to the other two cell lines (5-6 days). Visvesvara *et al*. have reported the use of monkey kidney cells (E6) and Hep-2 cells for growing *B. mandrillaris* but further discussion on the preferred cell line or cell count were not mentioned (Visvesvara *et al.*, 1990a). In this study, the MA104 cells were chosen for growing large-scale of *B. mandrillaris* because it was less laborious compared to the EA.hy926 which needed to be given frequently to the amoeba for production of high numbers of *B. mandrillaris*.

Contact between amoeba and mammalian cells is necessary to induce cell destruction (Kiderlen *et al.*, 2006, Matin *et al.*, 2006b). *B. mandrillaris* can feed on mammalian cells using several modes, including engulfment and penetration into the cells (Dunnebacke, 2007, Kiderlen *et al.*, 2006). It has been suggested that the release of certain enzymes (proteases) from the amoeba might be involved in the degradation of the cellular contents of the mammalian cells (Dunnebacke, 2007, Matin *et al.*, 2006b).

Dunnebacke has reported that feeding through penetration of amoeba into cells was more frequently seen rather than engulfment (Dunnebacke, 2007). In the study, it was shown that the initial sign of contact could be noticed by the presence of clear vesicles or small holes in mammalian cells that have been described as due to the ends of the *B. mandrillaris* pseudopodia. Small finger-like projections may emerge from the pseudopodia and function to trap smaller pieces of the target cell for ingestion by the pseudopodia (Dunnebacke, 2007). This similar piecemeal engulfment activity on mammalian cells has also been shown by *N. fowleri* and has been described as "trogocytosis" (Brown, 1979). Additionally, feeding by *B. mandrillaris* also can involve penetration of the entire amoeba into the target cell (Dunnebacke, 2007). The cytoplasmic components are the first to be consumed by the amoeba and eventually the nucleus. Hence, the mammalian cells would maintain their integrity for days before disruption or clearance of the intact monolayer could be seen (Dunnebacke, 2007). Similar feeding activity events were also seen in this study, as reported in Section 3.2.1 and Table 3.1. Here, although damaged mammalian cells, as shown by the rounded morphology of the cells, were observed, the monolayer remained intact for the first 24-48 hrs depending on the cell line. This was eventually followed by gradual clearance of the monolayer and increased *B. mandrillaris* numbers. In addition, the presence of small holes on mammalian cells and the penetration of an amoeba into a mammalian cell, as described by Dunnebacke (2007), was also observed in this study.

As an alternative to mammalian cells, *Acanthamoeba* spp. and *Naegleria* spp. have also been used to aid in isolation of *B. mandrillaris* from clinical and environmental samples (Schuster *et al.*, 2003, Visvesvara *et al.*, 1993). In agreement, the results in Sections 3.2.2.1 and 3.2.2.2 showed that *B. mandrillaris* was capable of feeding on both *A. castellanii* and *N. gruberi*. Here, the response of *B. mandrillaris* to both amoebae when

used as the food source was determined by measuring the distance *B. mandrillaris* migrated from the starting point of NNA and NNA-*E. coli* plates that had been lawned with single-type of amoeba or combination of both *Acanthamoeba* or *Naegleria*. The results showed that the distance of *B. mandrillaris* migration regardless what amoeba was used, was farther on NNA rather than on NNA-*E. coli* plates. A possible explanation for this would be that on the NNA plates, there was no food source for *Acanthamoeba* and *Naegleria*. Therefore, this unfavourable condition might hinder the proliferation of both of the amoebae. As a consequence, if *B. mandrillaris* were to feed on these amoebae they must travel from the starting point to areas with high numbers of *Acanthamoeba* or *Naegleria* once they have consumed those two amoebae that were present near to the inoculation point. In contrast, on NNA-*E. coli* plates, the *Acanthamoeba* and *Naegleria* could feed on *E. coli* and actively proliferate all over the plates. Hence, there would be an abundant food source for *B. mandrillaris* and the migration on the plate would be less. This was clearly shown by the morphology of the *B. mandrillaris* trophozoites on NNA-*E. coli* lawned with *Acanthamoeba* plates, which appeared to have thick branches and be less elongated compared to those seen on NNA plates lawned with *Acanthamoeba* alone. It was also noticed that *B. mandrillaris* preferred *Acanthamoeba* trophozoites rather than the cysts, as shown by the present of high numbers of *Acanthamoeba* cysts at the end of the experiment although abundant of *B. mandrillaris* were present in close vicinity. Probably, this limited feeding activity on *Acanthamoeba* cysts could be due to the double-cell wall of the cysts (Marciano-Cabral *et al.*, 2003a) which protected them from being punctured or invaded by the *B. mandrillaris*. In addition, the *Acanthamoeba* cysts might be difficult to degrade as the cyst wall has been reported to comprise of polysaccharides (particularly cellulose) that is lacking in the trophozoite stage (Neff *et al.*, 1969, Weisman, 1976). Further support

of our observation comes from another study which showed that *Acanthamoeba* cysts were not consumed by *B. mandrillaris* even after 10 days of co-culture (Matin *et al.*, 2006a).

In this study, *B. mandrillaris* was also observed to show preference for *A. castellanii* rather than *N. gruberi*. As *Naegleria* moves faster than *Acanthamoeba* it would make more difficult for *Balamuthia* to feed on them. According to the literature, depending on the strains of *Naegleria* the movement can reach up to 1.67 μms^{-1} as compared to ≤ 0.42 μms^{-1} for *Acanthamoeba* and 0.24 μms^{-1} for *B. mandrillaris* (Fulton, 1977, Schuster *et al.*, 2008a). Besides that, *Naegleria* was seen to be sensitive to dry conditions (Chang, 1978) and food deprivation, which may explain the numbers of dead amoebae towards the end of the experiments. Previously, Chang (1978) has showed that suspensions of *Naegleria* trophozoites that were left to dry on slides would died instantly. In addition to dry condition, others have reported that *Naegleria* is more sensitive than *Acanthamoeba* and *Balamuthia* to culture conditions including pH changes, waste accumulation and frequent subculturing is needed to avoid degradation of the amoeba (Schuster, 2002). Init *et al*. (2010) have suggested that *Naegleria* prefers watery areas for growth and proliferation. This is because in watery environments the amoeba is able to transform into a flagellate stage, which allows them to swim or move around to areas with abundant of food source (Schuster *et al.*, 2004c). Like *Acanthamoeba*, *Naegleria* trophozoites can transform into the cyst stage when the conditions are not appropriate for survival (Marciano-Cabral, 1988). However, the cysts are delicate and more vulnerable to desiccation than those of *Acanthamoeba* (Init *et al.*, 2010). For example, *Naegleria* cysts that have been exposed to drying were seen to be non-viable in less than 5 minutes (Chang, 1978). In contrast, *Acanthamoeba* cysts are more resistant to harsh

conditions and can maintain their viability even after more than 20 years of desiccation (Sriram *et al.*, 2008).

Since *B. mandrillaris* has been grouped as free-living amoeba together with other common FLA e.g. *Acanthamoeba* spp. and *Naegleria* spp. that are ubiquitous in nature, it was thought that *B. mandrillaris* would have similar food requirement for growth as them. In the environment, free-living amoebae are capable of feeding on bacteria, fungi, yeasts algae and even other protozoa (Rodriguez-Zaragoza, 1994). This appears not to be the case for *B. mandrillaris* as it has been documented that *B. mandrillaris* does not feed on either Gram positive or Gram negative bacteria (Schuster, 2002, Matin *et al.*, 2006a). In this study, besides testing both types of bacteria as the food source, a fungus *Candida albicans* was also tested in parallel. The finding that showed *B. mandrillaris* only travelled $\leq$ 1.5 cm from the starting point confirms their lower preference towards bacteria or fungi as a food source. Recently, it has been reported that bacteria could support *B. mandrillaris* to maintain their trophozoite stage for up to 13 days, as compared to the control *B. mandrillaris* which changed into immature cysts within 24 hrs. In addition, the low preference of *B. mandrillaris* for bacteria uptake has been demonstrated using FITC-labelled *E. coli* K-12, with only some amoebae being seen to contain the labelled bacteria (Matin *et al.*, 2006a).

In the lab, both environmental strains of *Naegleria* and *Acanthamoeba* could be easily isolated and cultured using the conventional non-nutrient agar plates seeded with *E. coli* as the food source (Schuster, 2002). Although *B.mandrillaris* does not feed on bacteria, three successful isolations of the amoeba from environmental samples have been reported using the conventional technique (Dunnebacke *et al.*, 2003, Dunnebacke *et al.*, 2004, Niyyati *et al.*, 2009, Schuster *et al.*, 2003). However, the isolation of the amoeba is a tedious process and requires regular microscopic observations because *B.*

mandrillaris can only be detected following several weeks of incubation possibly as a result of feeding on other FLA that may present in the culture plates (Schuster *et al.*, 2003). In addition, *B. mandrillaris* has long doubling times (20-30 hrs) and therefore their growth on NNA-*E. coli* plates might be impeded by other organisms or contaminating fungi, resulting in unsuccessful isolation (Rideout *et al.*, 1997, Schuster *et al.*, 1996, Schuster *et al.*, 2003, Visvesvara *et al.*, 1990a).

In support of the conclusion of Visvesvara *et al*. (1993), the persistence of *B. mandrillaris* in the environment is believed not to be due to grazing on bacteria but mainly through feeding on other smaller protozoa including free-living amoebae such as *Acanthamoeba* and *Naegleria*.

4 Development of a DNA extraction method for environmental samples

4.1 Introduction

Due to the limited preference of *B. mandrillaris* towards bacteria as a food source, the isolation method using non-nutrient agar plates seeded with *E. coli* (NNA-*E. coli*) is not suitable for use in studying the ecological distribution of the amoeba (Schuster, 2002, Matin *et al.*, 2006a). Since its first isolation in 1986, only three environmental strains have been successfully isolated using this technique (Dunnebacke *et al.*, 2003, Schuster *et al.*, 2003, Niyyati *et al.*, 2009). Accordingly, molecular approaches without needing initial culturing might be of great advantage to overcome this situation. Previous studies have reported on the detection of uncultivable microorganisms using total DNA extracted directly from environmental samples and PCR (Amann *et al.*, 1995, Yeates *et al.*, 1997). Therefore this approach was tested for use with free-living amoebae.

Methods that have commonly been employed for direct DNA extraction include cell disruption by bead-beating or sonication, chemical lysis using detergent-containing mixtures and treatments with proteinase K or lysozyme (Carrigg *et al.*, 2007, Courtois *et al.*, 2001, Fortin *et al.*, 2004, Miller *et al.*, 1999, Ogram *et al.*, 1987, Schneegurt *et al.*, 2003, Steffan *et al.*, 1988). Nevertheless, to obtain an inhibitor-free DNA template is very challenging because direct DNA extracts from environmental samples are frequently contaminated with humic substances that may hinder DNA amplification by PCR (Zhou *et al.*, 1996). Thus, the extracted DNA needs to be further treated, such as with hexadecyltrimethylammonium bromide (CTAB) or polyvinylpolypyrrolidone (PVPP) (Zhou *et al.*, 1996, Ogram, 1998). In this part of the study, a direct DNA extraction method for use with environmental samples was developed without the need

of primary culturing on NNA-*E. coli* plates and the extracted DNA was tested for its suitability for molecular study.

4.2 Results

4.2.1 Optimisation of DNA yield from the UNSET-PEG method

In order to determine the efficiency of the DNA extraction method, small-scale (1 g) soil samples were subjected to the UNSET-PEG method (Section 2.18.1) and evaluations were done by agarose gel electrophoresis and spectrophotometry. The three parameters investigated were: sizes of glass beads, mechanical cell disruption and requirement for a phenol-chloroform extraction step for the removal of protein contamination. As can be seen in Figure 4.1, the yield of DNA was better with a combination of either 0.25 – 0.5 mm (Lane 2) or 0.5 – 1.0 mm beads (Lane 4) and ribolyser treatment rather than when combined with a vortex (Lanes 6 and 8). In addition, Figure 4.2 shows that the addition of a phenol/chloroform (P/C) step (Lanes 2 to 6) produced high quality (intact fragments) DNA with less DNA smearing than the DNA obtained from samples processed without the P/C step (Lanes 7 to 11). The DNA extracts without P/C appeared as darker brown than those which included the P/C step (results not shown). In addition, a 'cloud' was observed at the bottom part of the agarose gel in lanes 7 and 9 with samples not treated with P/C, suggested the presence of humic acids (Smalla *et al.*, 1993). Hence, the best combination for extracting DNA from small scale-soil samples was the use of 0.25 – 0.5 mm glass beads with a ribolyser and the inclusion of a phenol-chloroform extraction step.

For DNA extraction on larger scale soil samples (10 g), the use of a ribolyser is not practicable. Therefore, for these samples an alternative method was derived with the mechanical cell lysis was done using a vortex. In addition, the effect of different times

(30 sec, 1 min and 2 min) for vortexing and the need for an incubation step (30 min) in UNSET buffer for maximum cell lysis was evaluated. The results are summarised in Table 4.1. Highest DNA recovery was obtained with 2 min of vortexing and the inclusion of the 30 min incubation step. However, the vortexing time for 2 min was not practical when processing large numbers of samples. Consequently, for large-scale soil samples, the best combination was the use of 0.25 – 0.5 mm glass beads, vortexing for 1 min and 30 min incubation in UNSET buffer, to allow maximum cell lysis, and inclusion of the phenol-chloroform step. The DNA yield from large-scale rich loam soil samples collected in the East Midlands, UK using the best combination is shown in Figure 4.3. Intact DNA fragment was seen for all the five soil samples tested (Figure 4.3) but the DNA yield varied from sample to sample (Table 4.2).

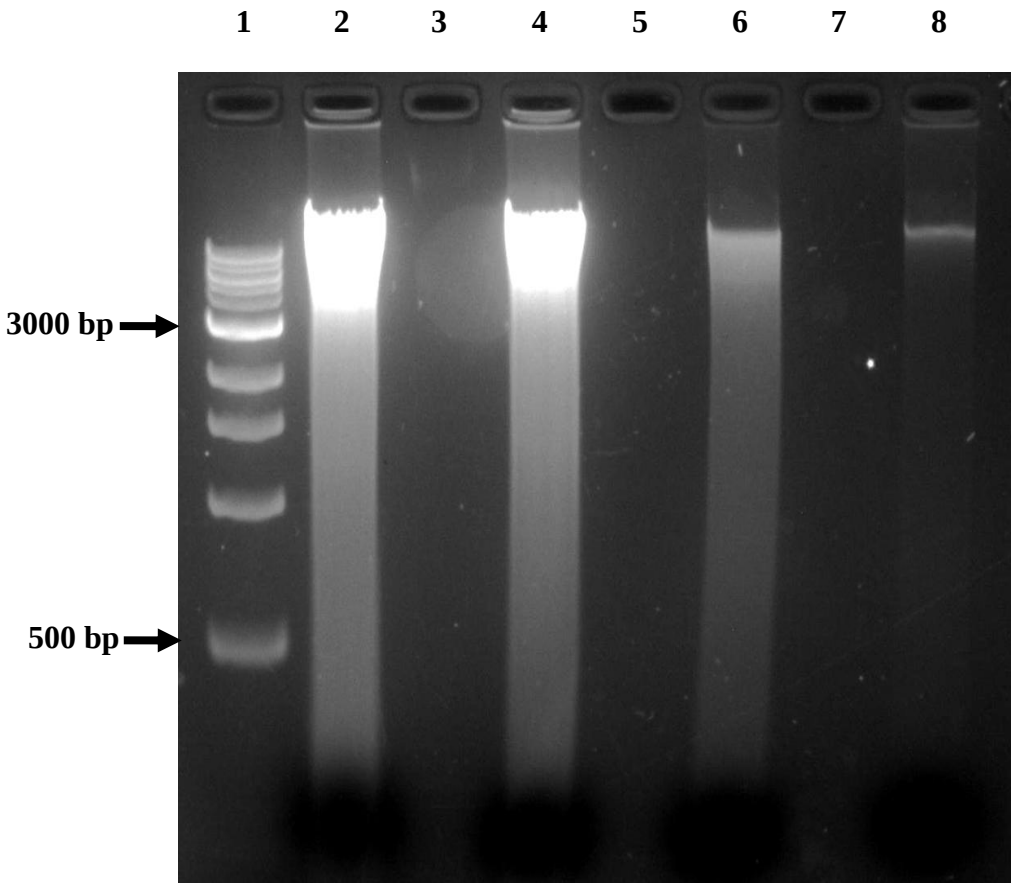


Figure 4.1 Effects of different sizes of glass beads and type of mechanical cell disruption on DNA yield from a small-scale soil sample (1 g). Lane 1: 1kbp marker; Lane 2: 0.25-0.5 mm glass beads plus ribolyser; Lane 4: 0.5-1.0 mm glass beads plus ribolyser; Lane 6: 0.25-0.5 mm glass beads plus vortex; Lane 8: 0.5-1.0 mm glass beads plus vortex

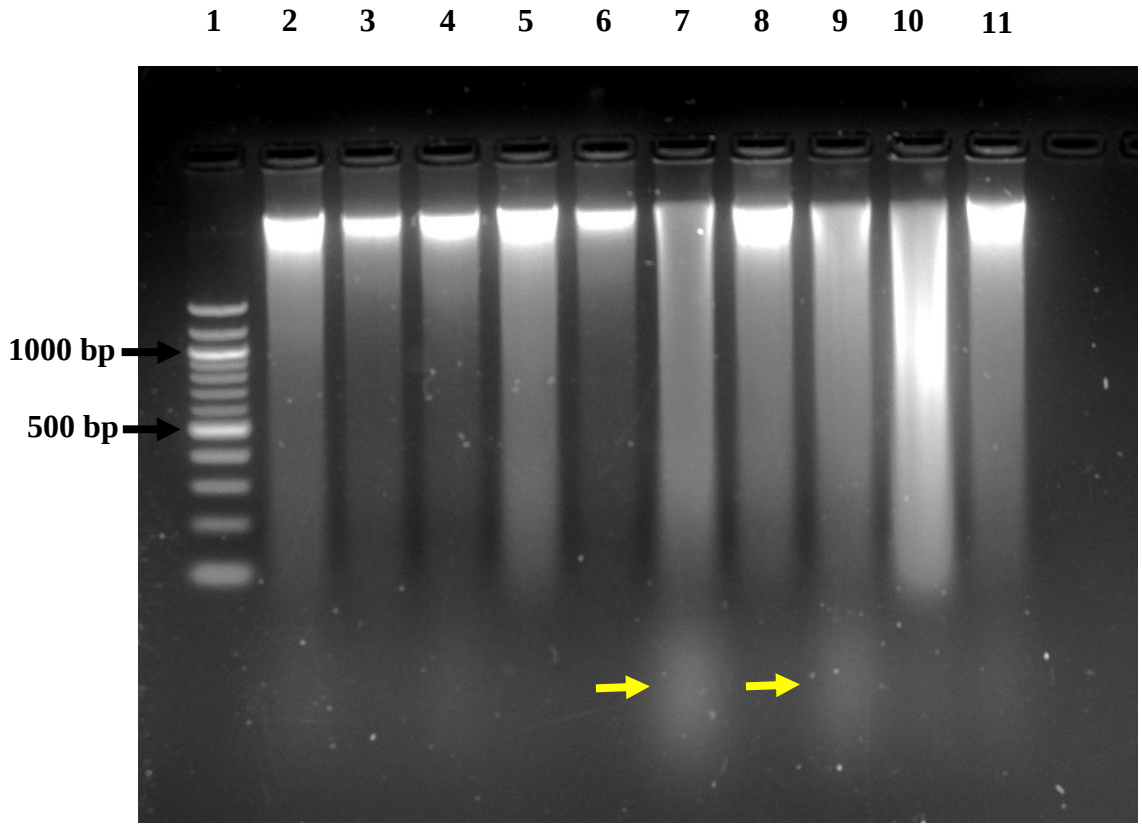


Figure 4.2 DNA yield with and without a phenol/chloroform (P/C) step (small-scale samples). Lane 1: 100 bp marker; Lane 2: EM08-03 (with P/C); Lane 3: EM08-04 (with P/C); Lane 4: EM08-05 (with P/C); Lane 5: EM08-06 (with P/C); Lane 6: EM08-07 (with P/C); Lane 7: EM08-03 (without P/C); Lane 8: EM08-04 (without P/C); Lane 9: EM08-05 (without P/C); Lane 10: EM08-06 (without P/C); Lane 11: EM08-07 (without P/C). Arrows shows the presence of humic acids (Smalla *et al.*, 1993). EM numbers represent soil samples collected from the East Midlands, UK (See Table 5.3).

Table 4.1 Effect of vortexing and incubation times on DNA yield from a large-scale soil samples

Incubation time	Vortexing time		
	30 sec	**1min**	**2 min**
0 min	±	+	++*
30 min	+	++	+++*

±: poor**; +:** moderate**; ++:** good**; +++:** best; *: more DNA degradation

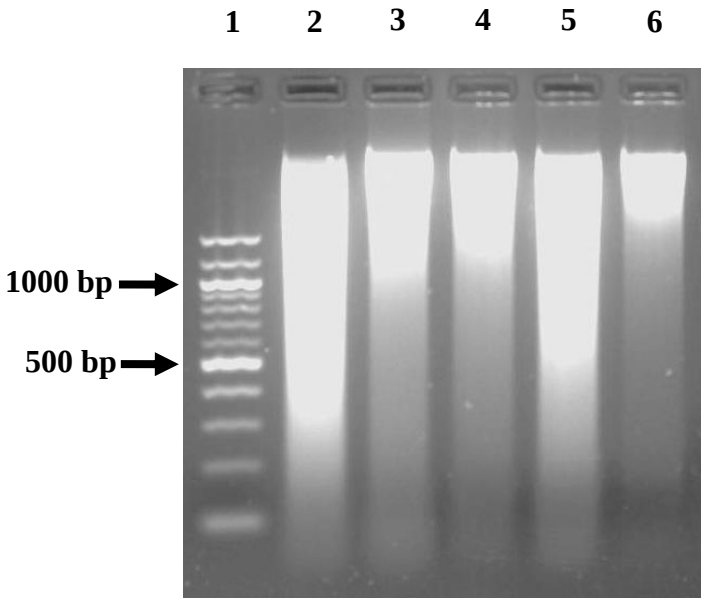


Figure 4.3 DNA yield from large-scale soil samples (10 g) using UNSET-PEG method with 0.25 – 0.5 mm glass beads, vortexing for 1 min followed by 30 min incubation in UNSET buffer and a phenol-chloroform step. Lane1: 100 bp marker; Lane 2: EM08-03; Lane 3: EM08-04; Lane 4: EM08-05; Lane 5: EM08-06; Lane 6: EM08-07. EM numbers represent soil samples collected in the East Midlands, UK (See Table 5.3).

Table 4.2 DNA yield by the UNSET-PEG method for 10 g soil samples

Sample Designation	DNA yield (µg/g)
EM08-03	19
EM08-04	11
EM08-05	10
EM08-06	18
EM08-07	8

EM numbers represent soil samples collected in the East Midlands, UK (See Table 5.3)

4.2.2 PCR for the detection of prokaryote DNA in crude DNA samples

The suitability of the DNA obtained in Section 4.2.1 for PCR was checked by amplifying the DNA using a modified prokaryotic 16S rDNA primer set. Results were recorded as positive when there was a band of the expected size or negative when no band was observed after the agarose gel electrophoresis. All undiluted small-scale soil samples that were subjected to PCR showed negative results. Dilution of the crude DNA to 10×, 100× and 1000× prior to PCR amplification also resulted negative results (Figure 4.4, Lane 2-4). The expected band of 500 bp (Lane 5) was seen only with the positive control using (*E. coli* DNA), while no band was obtained with the negative control. Therefore, the DNA was subjected to further DNA purification.

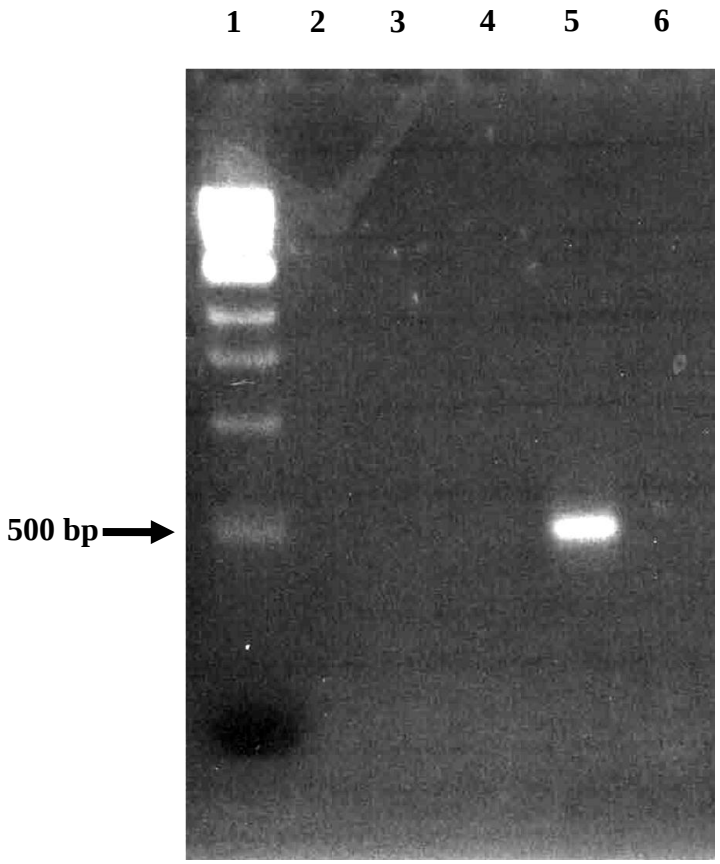


Figure 4.4 Amplification of bacterial 16S rDNA using different dilutions of DNA extracted from a small-scale soil sample (EM08-03). Lane 1: 1kbp marker; Lane 2: 10× dilution; Lane 3: 100× dilution; Lane 4: 1000× dilution; Lane 5: Positive control (*E. coli*); Lane 6: Negative control (nanopure water). Expected size of PCR product is 500 bp.

4.2.3 DNA yield after DNA purification

Purification using 10% (w/v) Chelex (Section 2.18.2.1) yielded negative results with the prokaryotic 16S rDNA PCR and a representative (EM08-03) is shown in Figure 4.5. Next, two types of DNA purification kit was tested for their effectiveness in removing contaminants from crude DNA obtained from the UNSET-PEG method. Results using small-scale samples (1 g) showed that the DNA yield after purification with the ZR soil microbe and Yorbio gel/ PCR methods were similar (Table 4.3) but a higher quality of

DNA was obtained from the ZR soil kit (Figure 4.6). Utilisation of the ZR soil kit for purification of crude DNA from five large-scale soil samples (10 g) resulted in good yield and high quality DNA, shown in Figure 4.7. The DNA yield for EM08-03, EM08-04, EM08-05, EM08-06 and EM08-07 were 2.1 µg/g, 1.3 µg/g, 1.5 µg/g, 1.6 µg/g and 1.8 µg/g respectively.

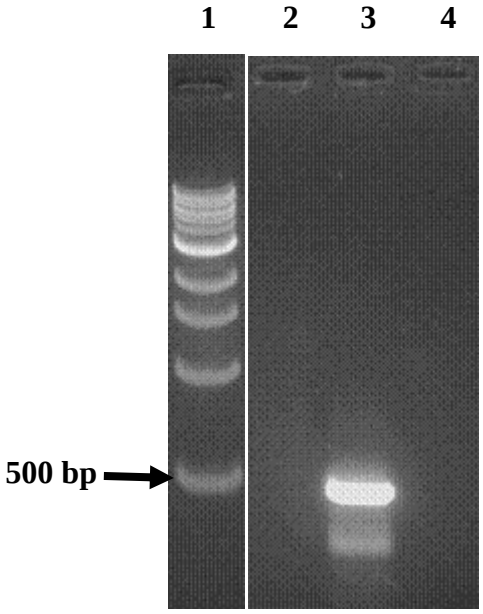


Figure 4.5 Amplification of bacterial 16S rDNA from a small-scale soil sample (EM08-03) after purification with 10% (w/v) Chelex. Lane 1: 1kbp marker; Lane 2: EM-08-03, Lane 3: Positive control (*E. coli*); Lane 4: Negative control (nanopure water). Expected size of PCR product is 500 bp.

Table 4.3 DNA yield after DNA purification from small-scale soil samples (1 g) with ZR soil microbe and Yorbio gel/PCR DNA kits

Environmental sample	DNA yield (µg/g)	
	ZR soil microbe kit	Yorbio gel/PCR DNA kit
EM08-03	7.2	7.9
EM08-04	7.3	7.0
EM08-05	6.0	6.0
EM08-06	4.1	3.7
EM08-07	5.2	5.2

EM numbers represent soil samples collected in the East Midlands, UK (See Table 5.3)

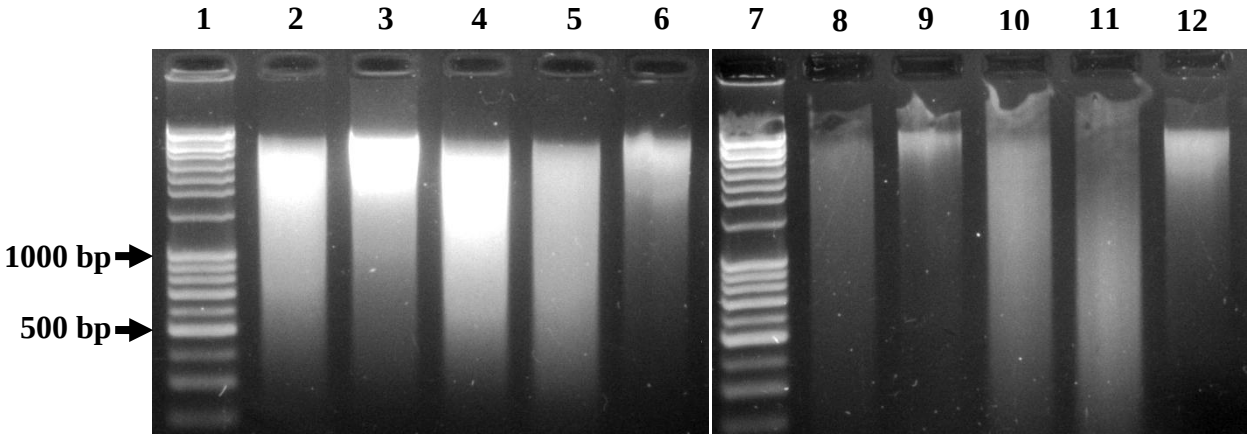


Figure 4.6 DNA yield for small-scale soil samples (1 g) after purification using a ZR soil microbe DNA kit (Z) or a Yorbio gel/ PCR DNA purification kit (Y). Lanes 1and 7: 1 kbp marker; Lane 2: EM08-03 (Z); Lane 3: EM08-04 (Z); Lane 4: EM08-05 (Z); Lane 5: EM08-06 (Z); Lane 6: EM08-07 (Z); Lane 8: EM08-03 (Y); Lane 9: EM08-04 (Y); Lane 10: EM08-05 (Y); Lane 11:EM08-06 (Y); Lane 12: EM08-07 (Y). EM numbers represent soil samples collected in the East Midlands, UK (See Table 5.3).

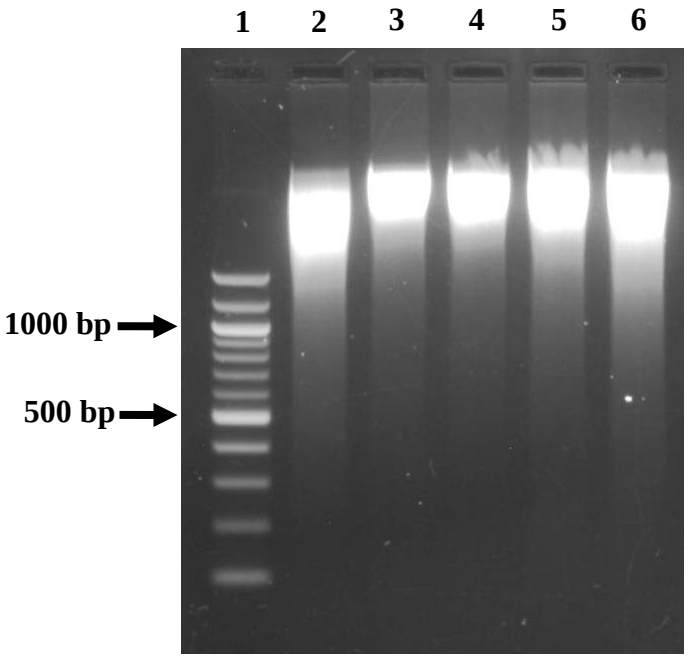


Figure 4.7 DNA yield for large-scale soil samples (10 g) after purification using ZR soil microbe DNA kit. Lane1: 100 bp marker; Lane 2: EM08-03; Lane 3: EM08-04; Lane 4: EM08-06; Lane 5: EM08-05; Lane 6: EM08-07. EM numbers represent soil samples collected in the East Midlands, UK (See Table 5.3).

4.2.4 PCR amplification of purified DNA with modified prokaryotic 16S rDNA primers

The capability of the purification kits to remove interfering contaminants from small-scale samples was assessed by PCR. Figure 4.8 shows that use of undiluted purified DNA from a representative soil sample, EM08-03 following treatment with Yorbio gel/PCR DNA purification kit, did not produce any PCR products of the expected 500 bp (Lanes 7 and 8). However, when purified DNA was diluted 1:20 (Lane 2), 1:50 (Lane 3), 1:100 (Lane 4), 1:1000 (Lane 5) and 1:5000 (Lane 6), bands with the expected size (500 bp) were seen. In contrast, purified DNA (small-scale samples) after treatment with the ZR soil microbe DNA kit could be used directly for 16S rDNA amplification

without the need for dilution (Figure 4.9). Similarly, all large-scale samples collected from the same areas as the small-scale samples gave a positive 16S rDNA amplification when used directly following purification with ZR soil microbe kit (Figure 4.10).

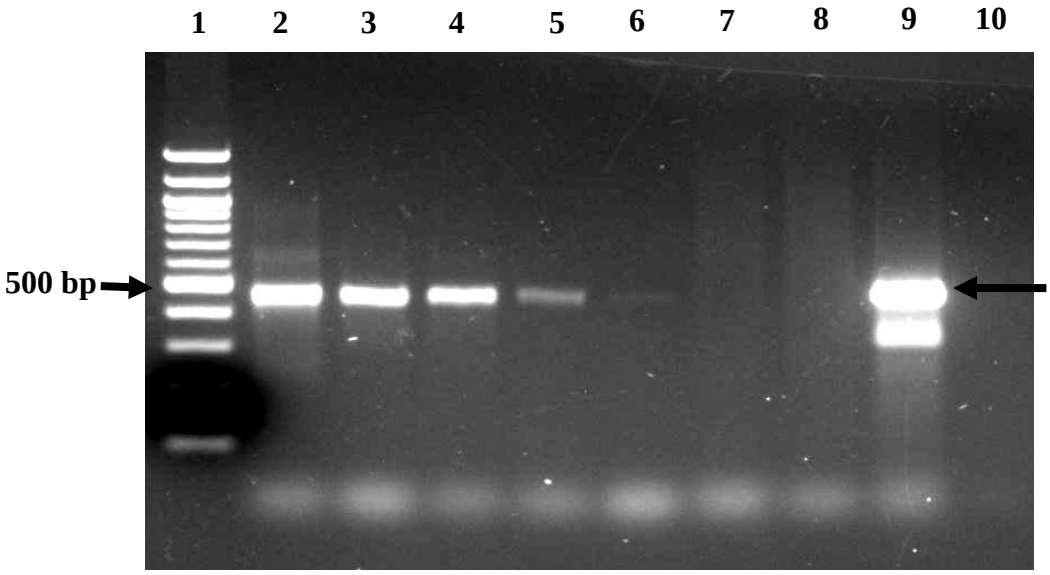


Figure 4.8 Amplification of bacterial 16S rDNA from a small-scale soil sample (EM08-03, 1 g) after purification with the Yorbio kit. Lane 1: 100 bp marker; Lane 2: Dilution 1:20; Lane 3: Dilution 1:50; Lane 4: Dilution 1:100; Lane 5: Dilution 1:1000; Lane 6: Dilution 1:5000; Lane 7: Without dilution (1 µl); Lane 8: Without dilution (4µl); Lane 9: Positive control (*E. coli*); Lane 10: Negative control (nanopure water). Expected size of PCR product is 500 bp (arrow).

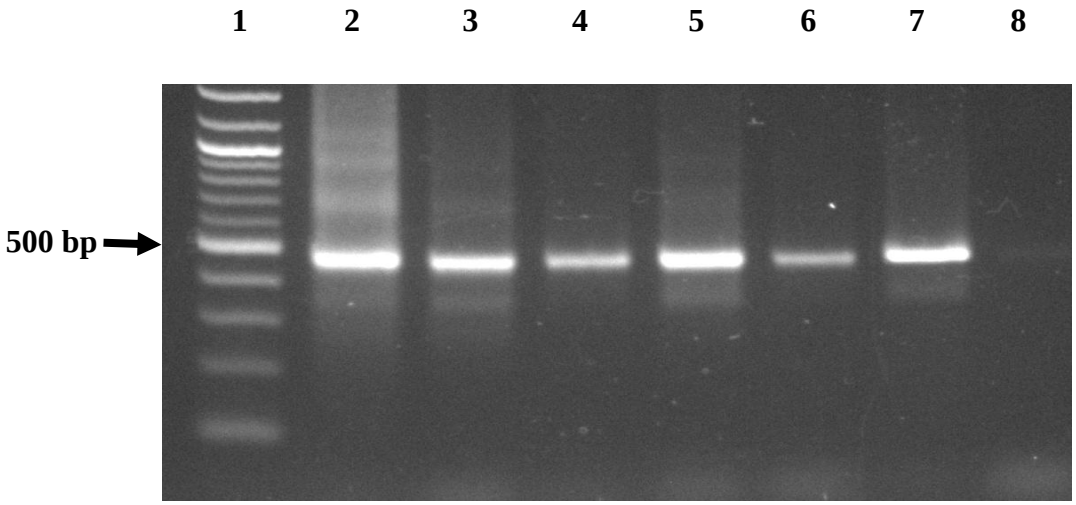


Figure 4.9 Amplification of bacterial 16S rDNA from undiluted small-scale soil samples (1 g) after purification with the ZR soil microbe DNA kit. Lane 1: 100 bp marker; Lane 2: EM08-03; Lane 3: EM08-04; Lane 4: EM08-05; Lane 5: EM08-06; Lane 6: EM08-07; Lane 7: Positive control (*E. coli*); Lane 8: Negative control (nanopure water). Expected size of PCR product is 500 bp. EM numbers represent soil samples collected in the East Midlands, UK (See Table 5.3).

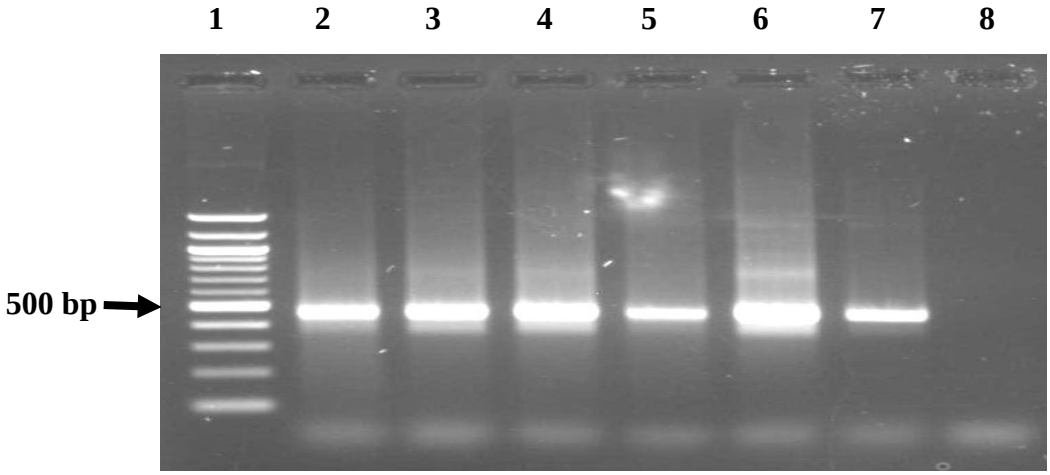


Figure 4.10 Amplification of bacterial 16S rDNA from undiluted large-scale soil samples (10 g) after purification with the ZR soil microbe DNA kit. Lane 1: 100 bp marker; Lane 2: EM08-03; Lane 3: EM08-04; Lane 4: EM08-05; Lane 5: EM08-06; Lane 6: EM08-07; Lane 7: Positive control (*E. coli*); Lane 8: Negative control (nanopure water). Expected size of PCR product is 500 bp. EM numbers represent soil samples collected in the East Midlands, UK (See Table 5.3).

4.3 Discussion

For the extraction of DNA from amoebae, the UNSET buffer is generally used in the free-living amoebae lab at Leicester University, including for *Acanthamoeba* spp., *Naegleria* spp. and other protozoa. However, for extracting DNA directly from environmental samples, chemicals including the 30% (w/v) polyethylene glycol in 1.6M NaCl, potassium acetate and phenol/chloroform have been incorporated to improve the purity of the extracted DNA. As bacteria are present in large numbers in soils, with as estimation of 10^9 bacteria in 1 g of soil (Holben, 1994), PCR using a universal prokaryotic 16S rDNA primers was used to test if extracted DNA was free from inhibitory levels of PCR inhibitors. The findings in Section 4.2.2 showed that although high concentrations of DNA were obtained, the extracted DNA yielded negative PCR results even after dilution up to 1000 times. This suggested the presence of PCR inhibitors, particularly humic substances as evidenced by the brown to black colour of the DNA extracts. In addition, the high DNA concentrations, as determined spectrophotometrically might actually be an artifact due to the presence of humic substances. This is because humic substances can affect DNA quantitation as they are able to absorb at 260 nm, which is commonly used to quantitate DNA (Smalla *et al.*, 1993, Holben, 1994).

It was assumed that most other contaminants, such as protein and polysaccharides from the environmental samples, had been removed during the UNSET-PEG method. This enabled further purification of the DNA extracts from large-scale soils (10 g) to be performed using the ZR soil microbe kit, which is actually designed to extract DNA up to 0.25 g of soil in each supplied column (Zymo Reasearch Corp.). In this study, instead of using the kit for DNA extraction, it has been employed as a secondary purification step and for that reason the cell lysis steps from the kit were omitted. The ZR soil

microbe DNA kit was chosen rather than the Yorbio gel/PCR DNA purification kit (Yorkshire Bioscience Ltd.) because of the advantage that the purifed DNA can be subjected to PCR directly, without the need for dilution. This is possibly because the final step of the kit involves the use of a column filled with a matrix (unknown) and a crosslinked polyvinylpyrrolidone (PVP) membrane. It is presumed that PVP was the beneficial aspect because previously, PVP which is a water-soluble polymer, has been incorporated into agarose gel for separation of DNA from humic substances (Young *et al.*, 1993, Ogram, 1998). Other chemicals that have been used to aid in the removal of humic substances and polysaccharide contamination include polyvinylpolypyrrolidone (PVPP) and hexadecyltrimethylammonium bromide (CTAB) (Ogram, 1998, Young *et al.*, 1993, Krsek *et al.*, 1999). It has been demonstrated that the use of PVPP was ineffective during cell lysis but efficient when used as a spin column during purification step (Krsek *et al.*, 1999). The application of the developed DNA extraction method for direct DNA extraction from soil and water samples collected from various geographical locations is shown in Chapter 5.

5 PCR for detection of free-living amoebae

5.1 Introduction

Having a suitable DNA extraction method that can be used in PCR (Chapter 4), the objective of this chapter was to proceed to the development of a specific PCR assay that can be used for studying the ecology and geographical distribution of *B. mandrillaris*, without requiring primary culturing of the amoeba on NNA-*E. coli* plates. To this end, a nested PCR assay was developed and tested for their value in rapid detection of *B. mandrillaris* from soil and water samples collected from various geographical locations when performed with the extracted DNA.

Although *N. fowleri* can be isolated using NNA-*E. coli* plates, an important consideration is that the growth of the amoeba may be suppressed by the presence of other thermophilic species, such as *N. lovaniensis* that grows at a faster rate than *N. fowleri* (Kilvington *et al.*, 1995a, Maclean *et al.*, 2004). A molecular approach using a nested PCR has been reported and applied for the detection of *N. fowleri* but it involves two separate reactions which can be laborious and prone to cross-contamination during transfer of primary PCR products for the secondary PCR reaction (Reveiller *et al.*, 2002). Hence, also in this chapter, the use of the developed DNA extraction method in combination with a *N. fowleri* one-step nested PCR was assessed for the ability to detect the presence of the amoeba in water samples. Finally, the suitability of the DNA extraction method for molecular detection of another eukaryote, i.e. *Acanthamoeba* spp., was also investigated.

5.2 Results

5.2.1 Development of a nested PCR for environmental detection of *B. mandrillaris*

5.2.1.1 Optimisation of PCR annealing temperature for *B. mandrillaris* primers

Two sets of primers were designed for the detection of *B. mandrillaris* DNA from environmental samples by a nested PCR (see Table 2.3 for details). The locations of the external (ExtF/R) and internal (IntF/R) primers on a *B. mandrillaris* partial 18S rDNA sequence are shown in Figure 5.1. Amplification of *B. mandrillaris* (ATCC 50209) DNA as the positive control with the external (ExtF/R) and internal (IntF/R) primer sets yielded PCR products of 403 bp (Figure 5.2) and 201 bp (Figure 5.3), respectively. An experiment was performed using gradient PCR to determine the best annealing temperature for both the *B. mandrillaris* ExtF/R and IntF/R primers. Observation of the agarose gel showed almost similar intensity of PCR product (Figure 5.2, Lanes 2 to 13) using the ExtF/R primers for temperatures between 55°C-61°C. The same was also seen for those amplified using the IntF/R primers at temperatures between 45°C to 55°C (Figure 5.3, Lanes 2 to 13). Hence, detection of *B. mandrillaris* from environmental samples by a nested PCR was performed at annealing temperatures of 56°C and 48°C for the ExtF/R and IntF/R primer pairs, respectively.

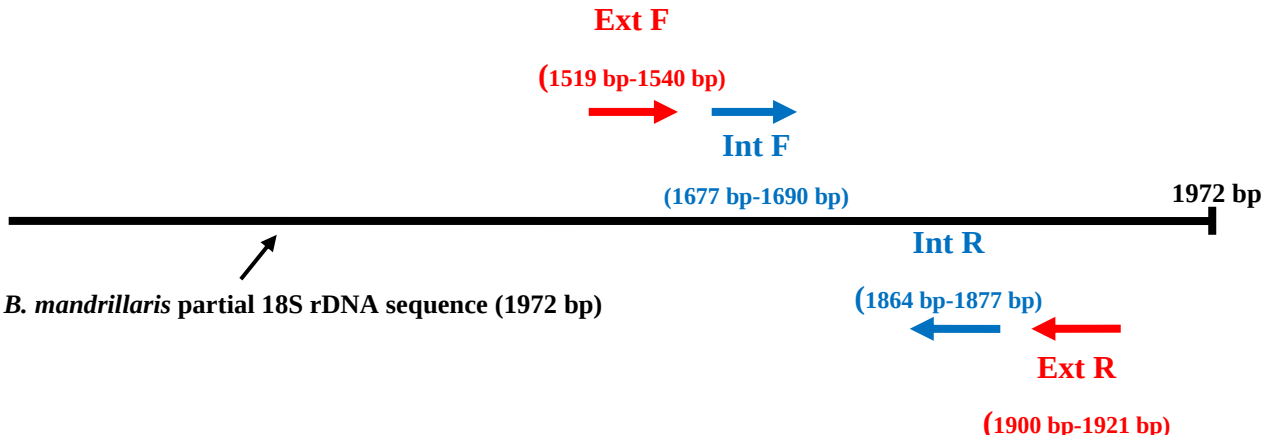


Figure 5.1 Diagram showing the locations of the external (Ext F/R) and internal primers (Int F/R) on a *B. mandrillaris* partial 18S rDNA sequence used in the development of nested PCR

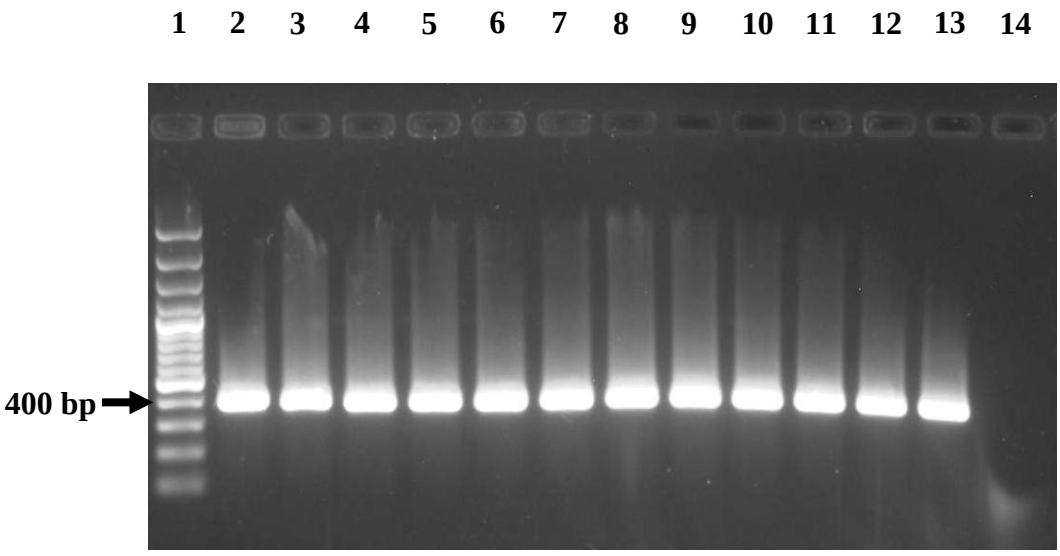


Figure 5.2 Optimisation of PCR annealing temperature for *B. mandrillaris* external primers. Lane 1: 100 bp marker; Lane 2: 55.0°C; Lane 3: 55.2°C; Lane 4: 55.6°C; Lane 5: 56.2°C; Lane 6: 56.9°C; Lane 7: 57.6°C; Lane 8: 58.4°C; Lane 9: 59.1°C; Lane 10: 59.8°C; Lane 11: 60.4°C; Lane 12: 60.8°C; Lane 13: 61.0°C; Lane 14: Negative control (nanopure water)

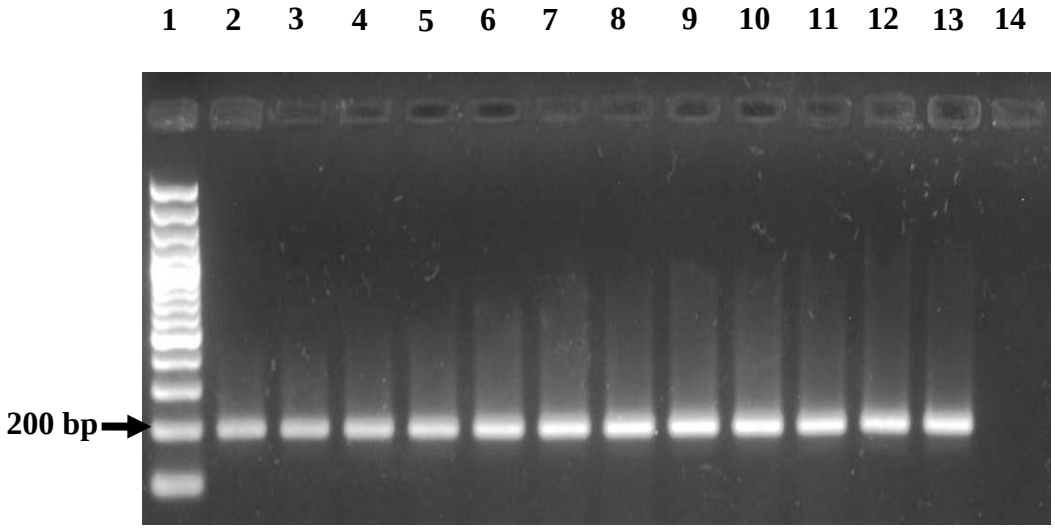


Figure 5.3 Optimisation of PCR annealing temperature for *B. mandrillaris* internal primers. Lane 1: 100 bp marker; Lane 2: 45.0°C; Lane 3: 45.3°C; Lane 4: 46.0°C; Lane 5: 47.0°C; Lane 6: 48.2°C; Lane 7: 49.4°C; Lane 8: 50.6°C; Lane 9: 51.8°C; Lane 10: 53.0°C; Lane 11: 54.0°C; Lane 12: 54.7°C; Lane 13: 55.0°C; Lane 14: Negative control (nanopure water)

5.2.1.2 Sensitivity of *B. mandrillaris* PCR

The sensitivity limit of *B. mandrillaris* primary and nested PCR was determined using soils spiked with *B. mandrillaris* trophozoites (see Section 2.19.2.1 for details). For this, soil samples, which were first shown to be negative for *B. mandrillaris* by the nested PCR, were spiked with different numbers of *B. mandrillaris* trophozoites and DNA was extracted using the method described previously (Section 2.18.3). PCR using the external primers alone showed a sensitivity limit of 100 trophozoites (Figure 5.4, Lane 2), while nested PCR following DNA reamplification using internal primers increased the sensitivity, with a band seen with soil spiked with 10 trophozoites (Figure 5.5, Lane 2). Subsequently, experiments were also performed using different amounts of *B. mandrillaris* genomic DNA. With the external primers alone, the brightest band (Figure

5.6, Lane 2) was seen with the highest amount of DNA (84.2 ng) tested, while the lowest amount of DNA that gave a PCR product of the expected size of 403 bp was 1 pg with a very faint band seen (Figure 5.6, Lane 7). Reamplification of primary PCR products with internal primers increased the sensitivity, with a single band of 201 bp seen with 100 fg of genomic DNA (Figure 5.7, Lane 8). High amounts of *B. mandrillaris* genomic DNA (10 pg to 84.2 ng) yielded two bands with size of 403 bp and 201 bp (Figure 5.7, Lanes 2 to 6). PCR reamplification with 1 pg of genomic DNA yielded a band at 201 bp (Figure 5.7, Lane 7).

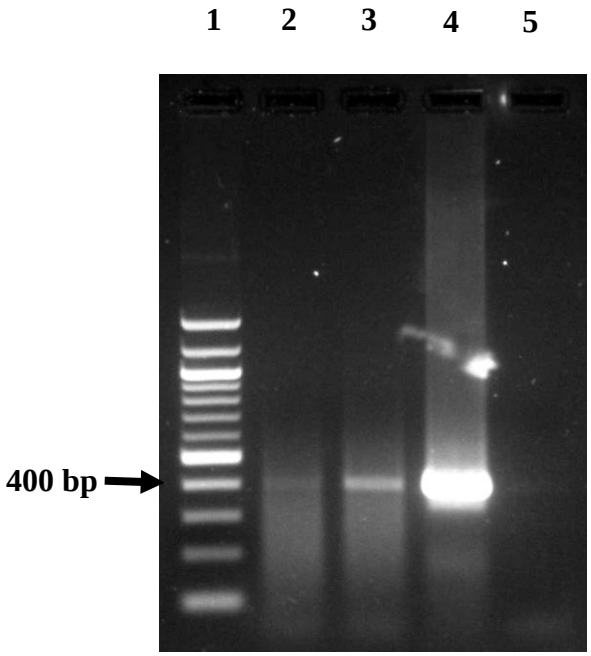


Figure 5.4 Sensitivity of *B. mandrillaris* PCR using only external primers (ExtF/R) with spiked soils. Lane 1: 100 bp marker; Lane 2: Soil spiked with 100 trophozoites; Lane 3: Soil spiked with 1000 trophozoites; Lane 4: Positive control (*B. mandrillaris* DNA); Lane 5: Negative control (nanopure water)

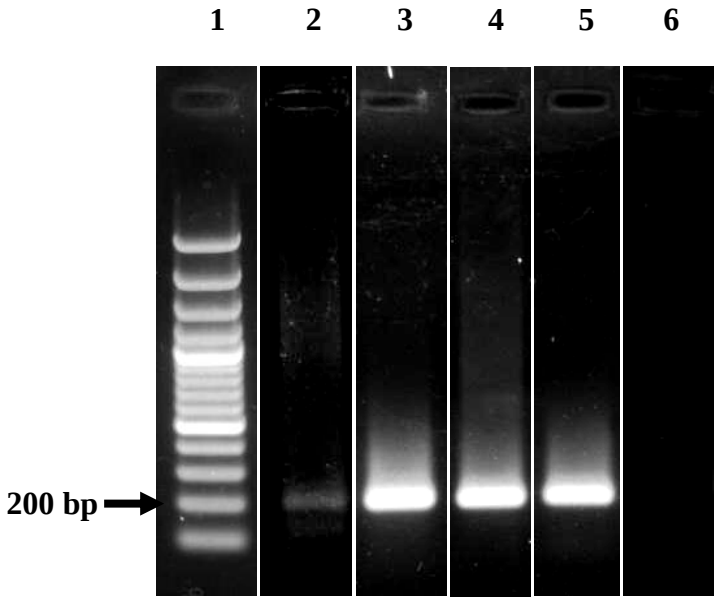


Figure 5.5 Sensitivity of *B. mandrillaris* nested PCR with spiked soils following DNA reamplification using internal primers (IntF/R). Lane 1: 100 bp marker; Lane 2: Soil spiked with 10 trophozoites; Lane 3: Soil spiked with 100 trophozoites; Lane 4: Soil spiked with 1000 trophozoites; Lane 5: Positive control (*B. mandrillaris* DNA); Lane 6: Negative control (nanopure water)

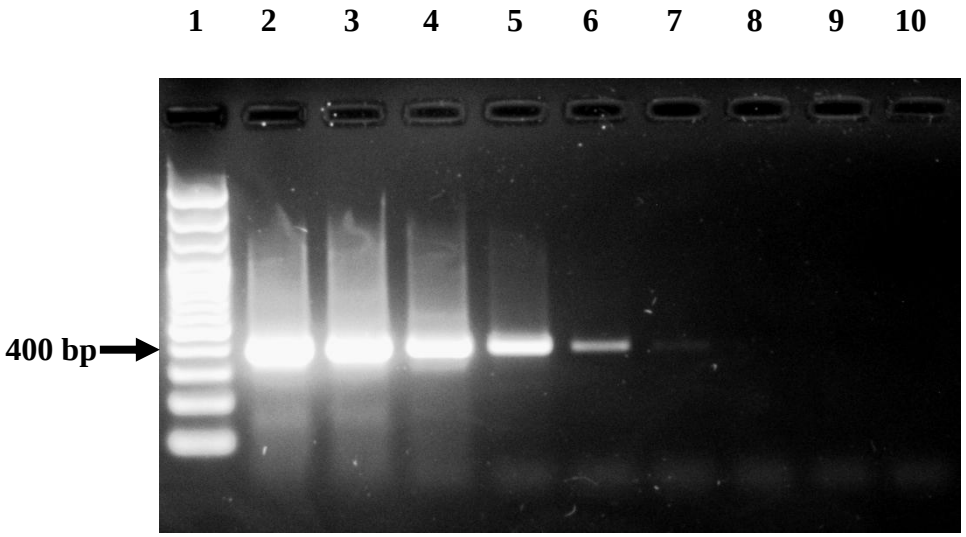


Figure 5.6 Sensitivity of *B. mandrillaris* PCR using only external primers (ExtF/R) with *B. mandrillaris* genomic DNA. Lane 1: 100 bp marker; Lane 2: 84.2 ng; Lane 3: 10 ng; Lane 4: 1 ng; Lane 5: 100 pg; Lane 6: 10 pg; Lane 7: 1 pg; Lane 8: 100 fg; Lane 9: 10 fg; Lane 10: Negative control (nanopure water)

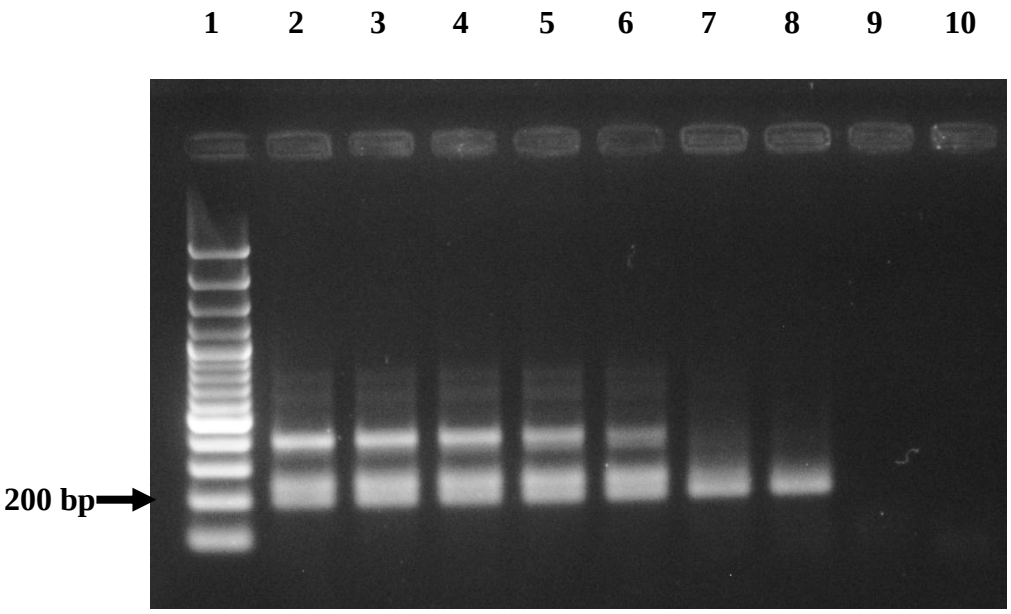


Figure 5.7 Sensitivity of *B. mandrillaris* nested PCR following reamplification of *B. mandrillaris* genomic DNA using internal primers (IntF/R). Lane 1: 100 bp marker; Lane 2: 84.2 ng; Lane 3: 10 ng; Lane 4: 1 ng; Lane 5: 100 pg; Lane 6: 10 pg; Lane 7: 1 pg; Lane 8: 100 fg; Lane 9: 10 fg; Lane 10: Negative control (nanopure water)

5.2.1.3 Specificity of *B. mandrillaris* PCR

PCR using the external primers (ExtF/R) alone or nested PCR following DNA reamplification using the internal primers (IntF/R) with genomic DNA from 15 *Acanthamoeba* spp. [*A. polyphaga* (CCAP 1501/3G), *A. polyphaga* (Ros), *A. polyphaga* (ATCC 30461), *A. castellanii* (ATCC 50370), *A. castellanii* (ATCC 30234), *A. hatchetti* (CDC V573), *Acanthamoeba* sp. (SCSK09-01), *Acanthamoeba* sp. (SCSK09-02), *Acanthamoeba* sp. (SCSK09-03), *Acanthamoeba* sp. (SCSK09-04), *Acanthamoeba* sp. (SCSK09-05), *Acanthamoeba* sp. (SCSK09-06), *Acanthamoeba* sp. (SCSK09-07), *Acanthamoeba* sp. (SCSK09-08) and *Acanthamoeba* sp. (SCSK09-09)], 4 *Naegleria* spp. [*N. lovaniensis* (C0490), *N. lovaniensis* (SK 41/44/1*), N. australiensis* (AB-T-F3) and *N. gruberi* (ATCC 30224)], *E.coli* (JM101 and JM109) and MA104 cells (ECCC

85102918) were negative. A single band with the expected size of 403 bp (ExtF/R) or 201 bp (IntF/R) was only seen when amplification was done using *B. mandrillaris* DNA (Figure 5.8 to Figure 5.10).

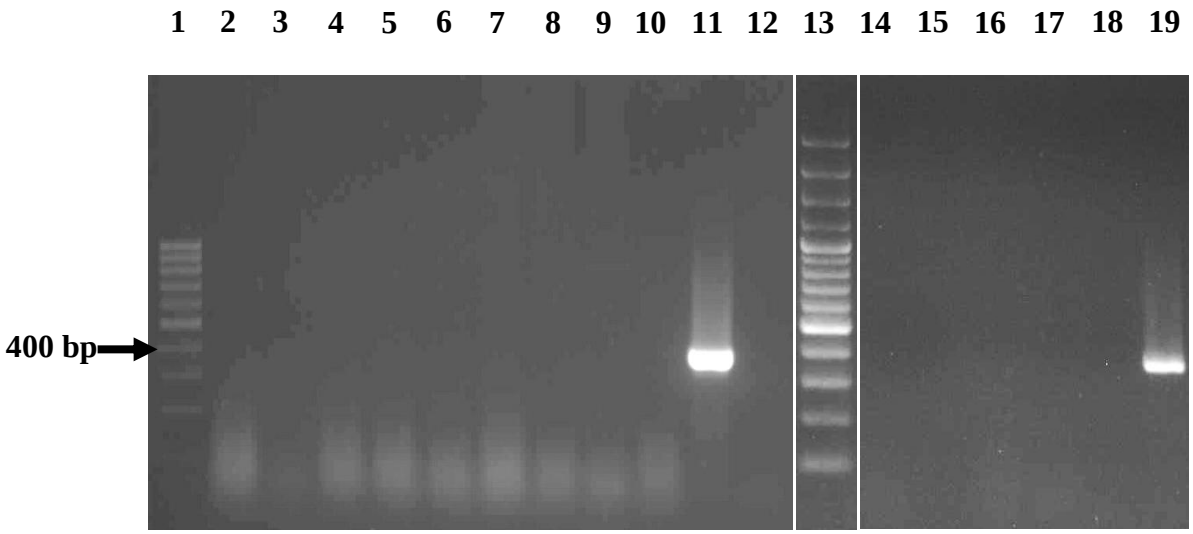


Figure 5.8 Specificity of *B. mandrillaris* PCR using only external primers (ExtF/R) against other prokaryotic and eukaryotic cells. Lanes 1 and 13: 100 bp marker; Lane 2: *A. polyphaga* (Ros strain); Lane 3: *A. hatchetti* (CDC V573); Lane 4: *A. castellanii* (ATCC 30234); Lane 5: *A. polyphaga* (CCAP 1501/3G; Lane 6: *A. castellanii* (ATCC 50370); Lane 7: *A. polyphaga* (ATCC 30461); Lane 8: *E. coli* (JM101); Lane 9: *E. coli* (JM109); Lane 10: *N. lovaniensis* (C0490); Lanes 11 and 19: *B. mandrillaris* DNA (ATCC 50209); Lane 12: Negative control (nanopure water); Lane 14: *N. lovaniensis* (SK 41/44/1), Lane 15: *N. australiensis* (AB-T-F3); Lane 16: *N. gruberi* (ATCC 30224); Lane 17: MA104 cells (ECCC 85102918); Lane 18: Negative control (nanopure water)

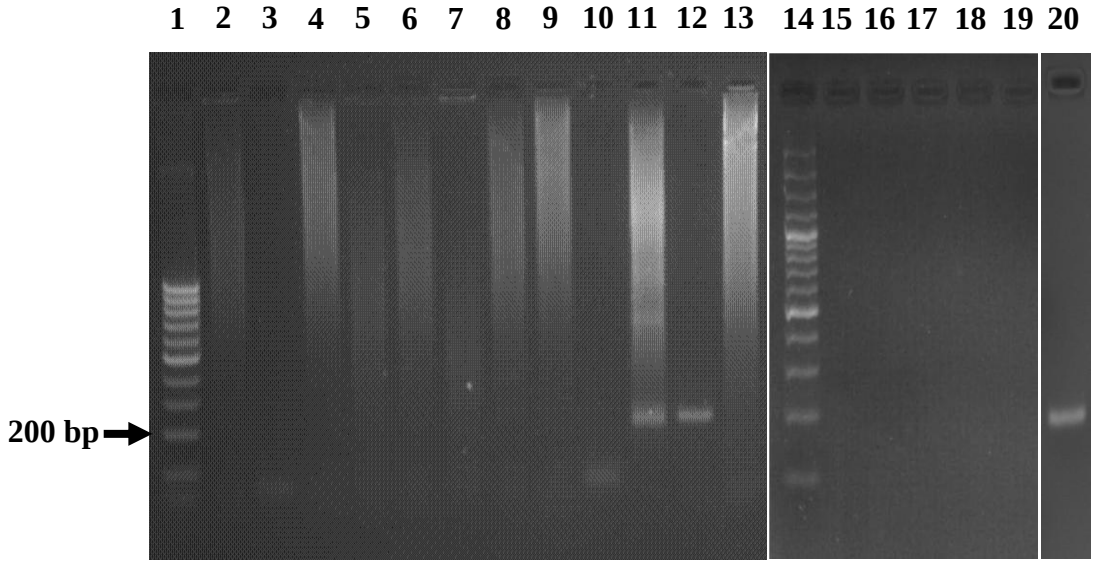


Figure 5.9 Specificity of *B. mandrillaris* nested PCR against other prokaryotic and eukaryotic cells following DNA reamplification using internal primers (IntF/R). Lanes 1 and 14: 100 bp marker; Lane 2: *A. polyphaga* (Ros strain); Lane 3: *A. hatchetti* (CDC V573); Lane 4: *A. castellanii* (ATCC 30234); Lane 5: *A. polyphaga* (CCAP 1501/3G); Lane 6: *A. castellanii* (ATCC 50370); Lane 7: *A. polyphaga* (ATCC 30461); Lane 8: *E. coli* (JM101); Lane 9: *E. coli* (JM109); Lane 10: *N. lovaniensis* (C0490); Lane 11: *B. mandrillaris* DNA (ATCC 50209) from primary PCR product; Lanes 12 and 20: *B. mandrillaris* DNA from primary PCR product (1:50); Lane 13: Negative control (nanopure water); Lane 15: *N. lovaniensis* (SK 41/44/1), Lane 16: *N. australiensis* (AB-T-F3); Lane 17: *N. gruberi* (ATCC 30224); Lane 18: MA104 cells (ECCC 85102918); Lane 19: Negative control (nanopure water)

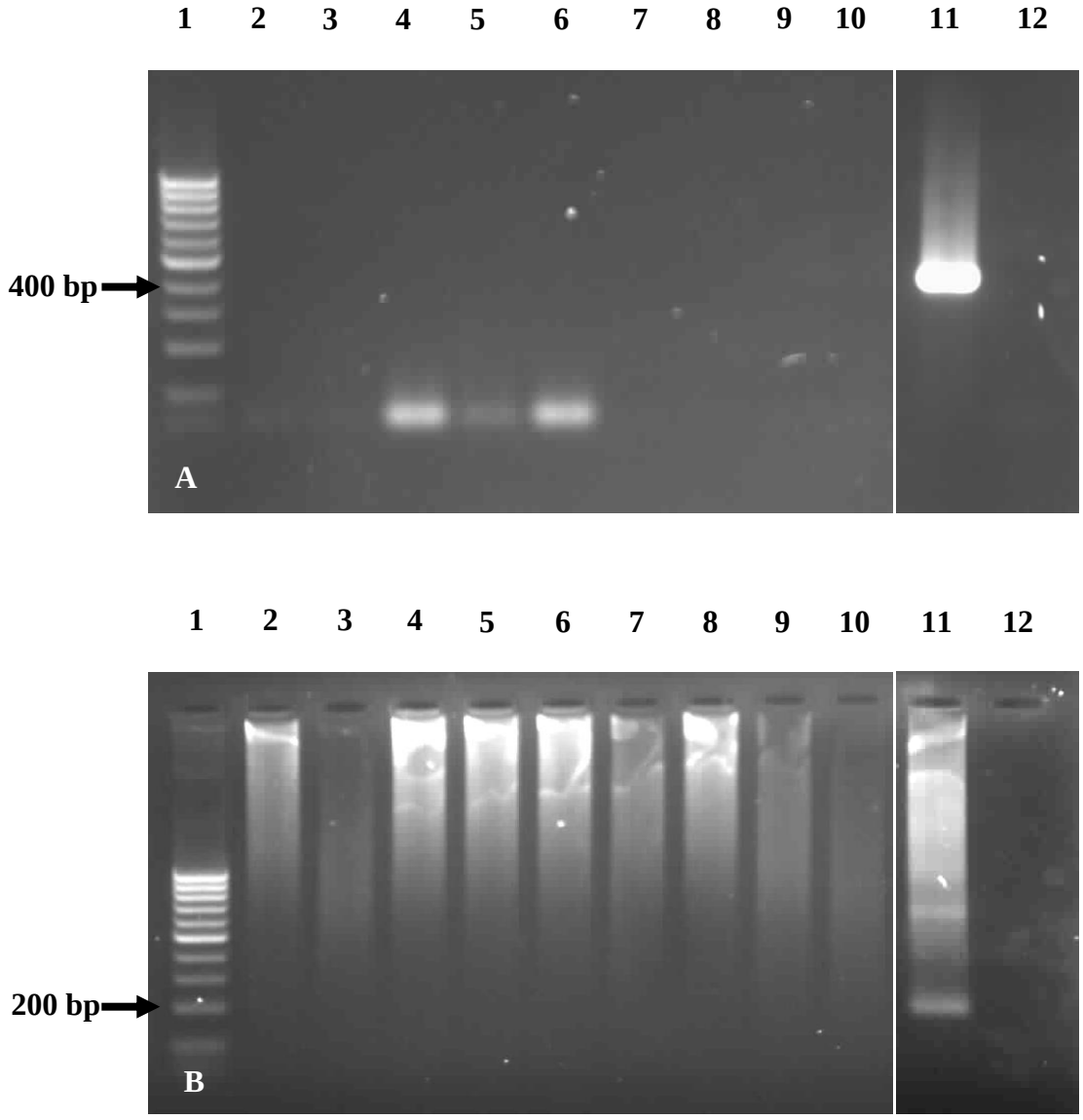


Figure 5.10 Specificity of *B. mandrillaris* PCR against *Acanthamoeba* isolates, using only external ExtF/R primers (A) and nested PCR following DNA reamplification using internal IntF/R (B). Lane 1: 100 bp marker; Lane 2: *Acanthamoeba* sp. (SCSK09-01); Lane 3: *Acanthamoeba* sp. (SCSK09-02); Lane 4: *Acanthamoeba* sp. (SCSK09-03); Lane 5: *Acanthamoeba* sp. (SCSK09-04); Lane 6: *Acanthamoeba* sp. (SCSK09-05); Lane 7: *Acanthamoeba* sp. (SCSK09-06); Lane 8: *Acanthamoeba* sp. (SCSK09-07); Lane 9: *Acanthamoeba* sp. (SCSK09-08); Lane 10: *Acanthamoeba* sp. (SCSK09-09; Lane 11: *B. mandrillaris* DNA; Lane 12: Negative control (nanopure water)

5.2.2 Detection of prokaryotes and *B. mandrillaris* DNA from environmental samples

PCR assays using bacterial 16S rDNA primers were performed to test the suitability of the purified DNA samples for subsequent PCR assays using primers for free-living amoebae. It was presumed that because bacterial DNA would be vastly in excess of protozoal DNA in environmental samples and that negative results after reamplification using the same bacterial primers would suggest the presence of PCR inhibitors and these samples should be excluded from further study. Out of 186 environmental samples tested, 167 (90%) were positive by PCR with the bacterial 16S rDNA primers. Of the 19 negative samples, all (100%) were positive on reamplification with the bacterial 16S rDNA primers. Primary PCR amplification of the DNA samples using the *B. mandrillaris* external primers alone yielded 17/186 (9%) positive results compared to 77/186 (41%) when nested PCR was used. These positive samples were 53% (58/109) clear water or water with sediment samples from France, 94% (16/17) soil samples from Southern Californian, 100% (2/2) from Republic of South Africa and 12.5% (1/8) from Portugal. All these nested PCR positives samples were negative for *B. mandrillaris* by cultivation on non-nutrient agar plates seeded with *E. coli*. None of the 44 samples of water, sediment or soil samples collected in the UK and 6 soils from Italy were positive for *B. mandrillaris* by the nested PCR or cultivation. Details of the results are discussed below and are subdivided based on geographical locations.

5.2.2.1 Water samples from River Tarn, South West France

A total of 109 water samples were collected from a river which receives thermal effluent from a power plant in France. Depending on the appearance of the water samples (clear/ cloudy), the samples were processed either by centrifugation or filtration prior to DNA extraction using the developed extraction method mentioned in Chapter 4.

The purified DNA yield showed variation between samples with ranged from 0.2 µg/750 ml to 21 µg/750 ml (Table 5.1). Visualisation on agarose gel showed from invisible (Lane 2) to visible (Lanes 3 to 7) DNA fragment as shown in Figure 5.11. PCR amplification using bacterial 16S rDNA primers yielded 98 positive amplifications (from 109 tests) and representatives are shown in Figure 5.12. The other 11 samples showed positive results only after a second round of PCR amplification and the PCR product for one of them (sample FW08-44) is shown in Figure 5.13. Ten of the samples were from clear water FW08-24, FW08-32, FW08-36, FW08-44, FW08-84, FW08-85, FW08-86, FW09-13, FW09-17 and FW09-18) and one from water with algae (FW08-26) (Table 5.1).

Detection of *B. mandrillaris* DNA using only the external primers (ExtF/R) showed only 13 positives results (Table 5.1). All showed PCR product of 403 bp when viewed on agarose gel and representatives are shown in Figure 5.14. Out of these, 11 (FW08-31, FW08-32, FW08-36, FW08-37, FW08-82, FW09-01, FW09-02, FW09-08, FW09-09, FW09-10 and FW09-11) were detected from clear water and 2 (FW08-19 and FW08-20) were from water with sediment. In contrast, the nested PCR showed 58 positives (Table 5.1). The majority of the positive samples were from clear water: a total of 37 (FW08-31, FW08-32, FW08-33, FW08-34, FW08-35, FW08-36, FW08-37, FW08-42, FW08-44, FW08-81, FW08-82, FW08-83, FW08-84, FW08-85, FW08-86, FW08-87, FW08-88, FW08-89, FW09-01, FW09-02, FW09-03, FW09-04, FW09-05, FW09-06, FW09-07, FW09-08, FW09-09, FW09-10, FW09-11, FW09-12, FW09-14, FW09-15, FW09-16, FW09-17, FW09-18, FW09-19 and FW09-20). These were followed with 16 samples from water with algae (FW08-45, FW08-46, FW08-47, FW08-48, FW08-49, FW08-50, FW08-52, FW08-53, FW08-55, FW08-56, FW08-57, FW08-59, FW08-60, FW08-77, FW08-79 and FW08-80), 3 from water with sediment

(FW08-19 and FW08-20 and FW08-90) and 2 from water with stones (FW08-54 and FW08-58). Representatives of the PCR positive samples using the nested PCR are shown in Figure 5.15. PCR products from FW08-19, FW08-20, FW08-32 and FW08-36 were sent for sequencing and results (Appendix 2) showed 100 % identity to *B. mandrillaris* DNA sequences deposited in the GenBank. *B. mandrillaris* was not detected from all the samples at both 32°C and 44°C incubations.

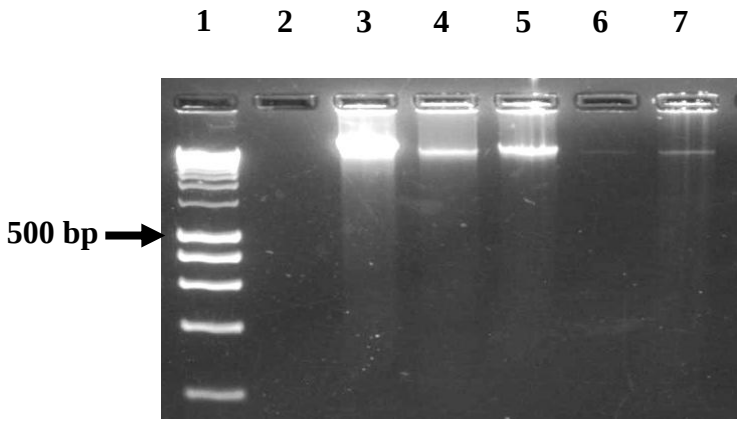


Figure 5.11 DNA yield after purification of River Tarn, South West France water samples with ZR soil kit. Lane 1: 1000 bp marker; Lane 2: FW08-44; Lane 3: FW08-45; Lane 4: FW08-46; Lane 5: FW08-47; Lane 6: FW08-48; Lane 7: FW08-49. FW numbers represent water samples collected in France (see Table 5.1)

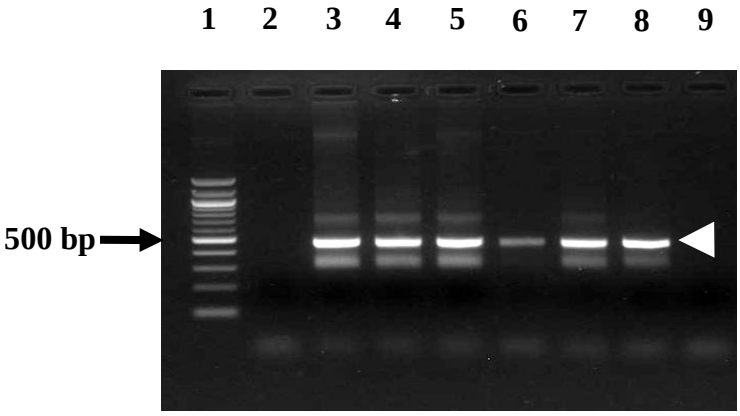


Figure 5.12 Bacterial 16S rDNA PCR with River Tarn, South West France water samples. Lane 1: 100 bp marker; Lane 2: FW08-44; Lane 3: FW08-45; Lane 4: FW08-46; Lane 5: FW08-47; Lane 6: FW08-48; Lane 7: FW08-49; Lane 8: Positive control (*E. coli* DNA); Lane 9: Negative control (nanopure water). White arrowhead shows the expected size (500 bp) of PCR product

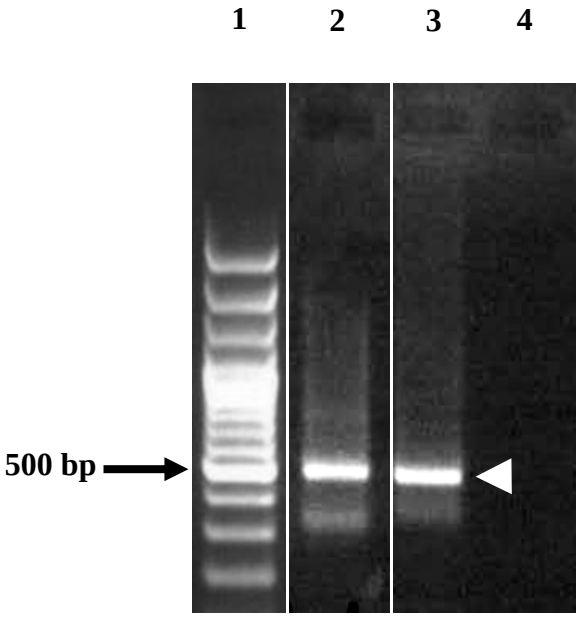


Figure 5.13 DNA Reamplification of sample FW08-44 with bacterial 16S rDNA primers. Lane 1: 100 bp marker; Lane 2: FW08-44; Lane 3: Positive control (*E. coli* DNA) Lane 4: Negative control (nanopure water). White arrowhead shows the expected size (500 bp) of PCR product

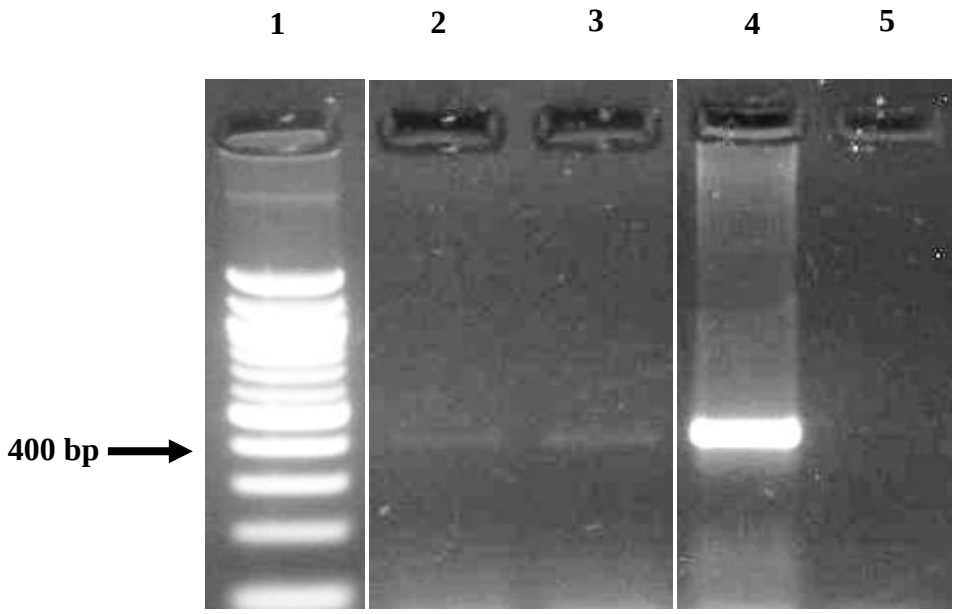


Figure 5.14 *Balamuthia mandrillaris* primary PCR using only external primers (ExtF/R) with River Tarn, South West France water samples. Lane 1: 100 bp marker; Lane 2: FW08-31; Lane 3: FW08-32; Lane 4: Positive control (*B. mandrillaris* DNA); Lane 5: Negative control (nanopure water)

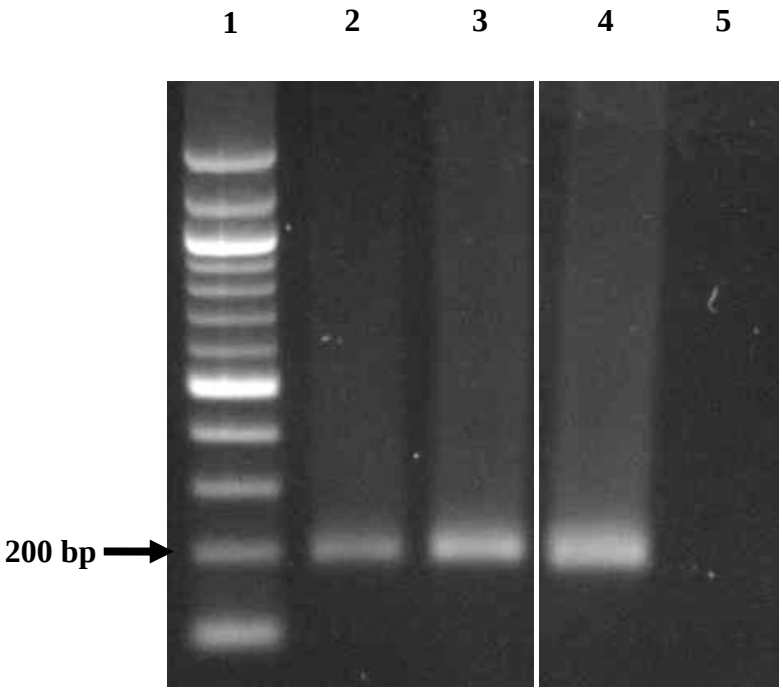


Figure 5.15 *Balamuthia mandrillaris* nested PCR with River Tarn, South West France water samples. Lane 1: 100 bp marker; Lane 2: FW08-31; Lane 3: FW08-32; Lane 4: Positive control (*B. mandrillaris* DNA); Lane 5: Negative control (nanopure water)

Table 5.1 Results of PCR assays and details for environmental samples collected from the River Tarn, South West France

Sample	Material	Total DNA yield (µg/750 ml)	Bacterial 16S rDNA PCR	*B. mandrillaris* 18S rDNA PCR	
				Ext F/R	Nested
FW08-19	H_2O+sediment	9.5	+	+	+
FW08-20	H_2O+sediment	9.9	+	+	+
FW08-21	H_2O	1.4	+	Neg	Neg
FW08-22	H_2O	1.2	+	Neg	Neg
FW08-23	H_2O	0.9	+	Neg	Neg
FW08-24	H_2O	1.0	Neg*	Neg	Neg
FW08-25	H_2O	1.3	+	Neg	Neg
FW08-26	H_2O+algae	1.7	Neg*	Neg	Neg
FW08-27	H_2O	1.1	+	Neg	Neg
FW08-28	H_2O	1.1	+	Neg	Neg
FW08-29	H_2O	0.8	+	Neg	Neg
FW08-30	H_2O+algae	2.2	+	Neg	Neg
FW08-31	H_2O	1.2	+	+	+
FW08-32	H_2O	0.6	Neg*	+	+
FW08-33	H_2O	1.3	+	Neg	+
FW08-34	H_2O	1.2	+	Neg	+
FW08-35	H_2O	1.1	+	Neg	+
FW08-36	H_2O	0.7	Neg*	+	+
FW08-37	H_2O	1.5	+	+	+
FW08-38	H_2O	2.0	+	Neg	Neg
FW08-39	H_2O	1.5	+	Neg	Neg
FW08-40	H_2O	1.1	+	Neg	Neg
FW08-41	H_2O	1.1	+	Neg	Neg
FW08-42	H_2O	1.2	+	Neg	+
FW08-43	H_2O	1.0	+	Neg	Neg
FW08-44	H_2O	0.4	Neg*	Neg	+

Continued:

FW08-45	H_2O+algae	5.4	+	Neg	+
FW08-46	H_2O+algae	2.7	+	Neg	+
FW08-47	H_2O+algae	3.6	+	Neg	+
FW08-48	H_2O+algae	1.1	+	Neg	+
FW08-49	H_2O+algae	1.4	+	Neg	+
FW08-50	H_2O+algae	2.3	+	Neg	+
FW08-51	H_2O+algae	15.4	+	Neg	Neg
FW08-52	H_2O+algae	4.5	+	Neg	+
FW08-53	H_2O+algae	5.2	+	Neg	+
FW08-54	H_2O+stones	2.2	+	Neg	+
FW08-55	H_2O+algae	0.9	+	Neg	+
FW08-56	H_2O+algae	1.1	+	Neg	+
FW08-57	H_2O+algae	4.7	+	Neg	+
FW08-58	H_2O+stones	3.0	+	Neg	+
FW08-59	H_2O+algae	3.9	+	Neg	+
FW08-60	H_2O+algae	3.6	+	Neg	+
FW08-61	H_2O+algae	1.8	+	Neg	Neg
FW08-62	H_2O+algae	1.6	+	Neg	Neg
FW08-63	H_2O	1.1	+	Neg	Neg
FW08-64	H_2O	1.2	+	Neg	Neg
FW08-65	H_2O	1.2	+	Neg	Neg
FW08-66	H_2O	1.7	+	Neg	Neg
FW08-67	H_2O	1.8	+	Neg	Neg
FW08-68	H_2O	2.5	+	Neg	Neg
FW08-69	H_2O	1.4	+	Neg	Neg
FW08-70	H_2O	1.7	+	Neg	Neg
FW08-71	H_2O	1.2	+	Neg	Neg
FW08-72	H_2O	1.0	+	Neg	Neg
FW08-73	H_2O	1.6	+	Neg	Neg
FW08-74	H_2O	1.8	+	Neg	Neg
FW08-75	H_2O	1.3	+	Neg	Neg

Continued:

FW08-76	H_2O+algae	2.1	+	Neg	Neg
FW08-77	H_2O+algae	2.4	+	Neg	+
FW08-79	H_2O+algae	4.6	+	Neg	+
FW08-80	H_2O+algae	2.3	+	Neg	+
FW08-81	H_2O	1.7	+	Neg	+
FW08-82	H_2O	2.4	+	+	+
FW08-83	H_2O	4.5	+	Neg	+
FW08-84	H_2O	0.6	Neg*	Neg	+
FW08-85	H_2O	0.7	Neg*	Neg	+
FW08-86	H_2O	0.6	Neg*	Neg	+
FW08-87	H_2O	1.2	+	Neg	+
FW08-88	H_2O	2.7	+	Neg	+
FW08-89	H_2O	1.5	+	Neg	+
FW08-90	H_2O+sediment	10.0	+	Neg	+
FW09-01	H_2O	1.0	+	+	+
FW09-02	H_2O	1.9	+	+	+
FW09-03	H_2O	2.1	+	Neg	+
FW09-04	H_2O	9.8	+	Neg	+
FW09-05	H_2O	20.1	+	Neg	+
FW09-06	H_2O	1.4	+	Neg	+
FW09-07	H_2O	3.9	+	Neg	+
FW09-08	H_2O	1.8	+	+	+
FW09-09	H_2O	0.2	+	+	+
FW09-10	H_2O	2.8	+	+	+
FW09-11	H_2O	1.0	+	+	+
FW09-12	H_2O	0.2	+	Neg	+
FW09-13	H_2O	0.2	Neg*	Neg	Neg
FW09-14	H_2O	2.9	+	Neg	+
FW09-15	H_2O	21.3	+	Neg	+
FW09-16	H_2O	0.2	+	Neg	+
FW09-17	H_2O	0.6	Neg*	Neg	+

Continued:

FW09-18	H_2O	0.7	Neg*	Neg	+
FW09-19	H_2O	5.0	+	Neg	+
FW09-20	H_2O	0.5	+	Neg	+
FW09-21	H_2O	1.1	+	Neg	Neg
FW09-22	H_2O	1.4	+	Neg	Neg
FW09-23	H_2O	1.1	+	Neg	Neg
FW09-24	H_2O	1.0	+	Neg	Neg
FW09-25	H_2O	0.7	+	Neg	Neg
FW09-26	H_2O	4.5	+	Neg	Neg
FW09-27	H_2O	1.8	+	Neg	Neg
FW09-28	H_2O+stones	6.6	+	Neg	Neg
FW09-29	H_2O+algae	1.7	+	Neg	Neg
FW09-30	H_2O	3.1	+	Neg	Neg
FW09-31	H_2O+algae	1.2	+	Neg	Neg
FW09-32	H_2O	1.9	+	Neg	Neg
FW09-33	H_2O+algae	1.5	+	Neg	Neg
FW09-34	H_2O	16.8	+	Neg	Neg
FW09-35	H_2O	0.3	+	Neg	Neg
FW09-36	H_2O+stones	0.4	+	Neg	Neg
FW09-37	H_2O+algae	7.0	+	Neg	Neg
FW09-38	H_2O	1.1	+	Neg	Neg

*positive after reamplification using the same bacterial 16S rDNA primers

5.2.2.2 Water samples from River Trent, Nottinghamshire, UK

As shown in the previous section (5.2.2.1) *B. mandrillaris* could be detected from a thermally polluted river in France. Therefore a similar experiment was conducted with samples from the River Trent, Nottinghamshire, UK. Ten (5 from the surface and 5 from the bottom) samples were collected from these different sites, as shown in Figure 5.16. Physical parameters were done at the field site and results were recorded (Table 5.2). All samples had a pH of 7.0 using the pH 1-11 indicator paper (Whatman, UK). The conductivity and temperature ranged from 0.53-0.68 mS/cm and 17.1-18.1°C respectively. All samples, except NT5 and NT7, showed dissolved oxygen between 7-9 mg/L by a Salifert O_2 kit. The dissolved oxygen for the later two could not be measured due to the samples being very muddy. As shown in Figure 5.17, DNA yields were higher from the samples taken from the river bottom (Lanes 2 to 6), except NT09-07 (Lane 5) compared to those collected from the surface of the river (Lanes 7 to 11) except NT09-08 (Lane 10). The quantitative measurements are shown in Table 5.2. All 10 samples were successfully amplified using the bacterial 16S rDNA primers (Figure 5.18). PCR amplification using either *B. mandrillaris* external primers alone (Figure 5.19) or nested primers (Figure 5.20) yielded negative results. None of the samples were positive for *B. mandrillaris* by cultivation at 32°C and 44°C. Free-living amoebae such as *Acanthamoeba*, *Cashia*, *Vahlkampfia*, *Naegleria*, *Hartmanella* and *Vanella* were detected from plates cultured at 32°C. At 44°C, amoebae seen were *Acanthamoeba*, *Vahlkampfia* and an unknown amoeba (Table 5.2).

Figure 5.16 Map showing sites (S1, S2 and S3) where water samples were collected from River Trent, Nottinghamshire, United Kingdom

Table 5.2 Physical parameters and cultivation results for River Trent, Nottinghamshire, United Kingdom water samples

Sample	**Area**	**pH**	**Dissolved oxygen (mg/L)**	**Conductivity (mS/cm)**	**Temperature (°C)**	**Total DNA yield (µg/750 ml)**	**Organisms cultured**	
							32°C	**44°C**
NT09-01	Bottom (S1)	7	7-9	0.62	17.1	5.5	*Vahlkampfia, Naegleria, Acanthamoeba, Vanella*	Unknown amoeba
NT09-02	Surface (S1)	7	7-9	0.56	17.1	1.3	*Acanthamoeba*	None
NT09-03	Bottom (S1)	7	7-9	0.61	17.1	2.7	*Acanthamoeba, Cashia, Hartmanella*	*Vahlkampfia*
NT09-04	Surface (S1)	7	7-9	0.60	17.1	1.7	Unknown amoeba	None
NT09-05	Bottom (S2)	7	Not available	0.67	18.1	20.1	*Vahlkampfia, Naegleria, Acanthamoeba*	Uknown amoeba
NT09-06	Surface (S2)	7	7-9	0.68	18.1	2.3	*Vanella, Hartmanella, Cashia*	None
NT09-07	Bottom (S3)	7	Not available	0.56	17.4	2.6	*Vahlkampfia, Acanthamoeba*	*Acanthamoeba, Vahlkampfia*
NT09-08	Surface (S3)	7	7-9	0.53	17.4	3.2	*Acanthamoeba*	Unknown amoeba
NT09-09	Surface (S3)	7	7-9	0.54	17.7	1.1	*Vahlkampfia, Acanthamoeba*	None
NT09-10	Bottom (S3)	7	7-9	0.54	17.7	15.8	*Vahlkampfia, Naegleria, Acanthamoeba, Hartmanella*	Uknown amoeba

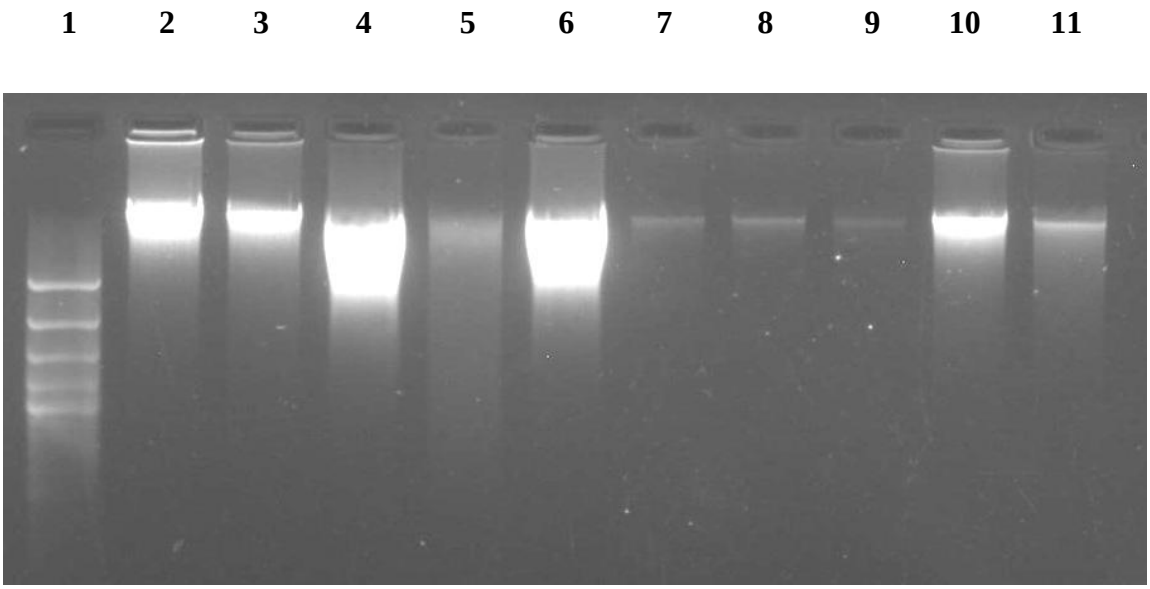


Figure 5.17 DNA yield after purification of River Trent water samples with ZR soil kit. Lane 1:100 bp marker; Lane 2: NT09-01; Lane 3: NT09-03; Lane 4: NT09-05; Lane 5: NT09-07; Lane 6: NT09-10; Lane 7: NT09-02; Lane 8: NT09-04; Lane 9: NT09-06; Lane 10: NT09-08; Lane 11: NT09-09

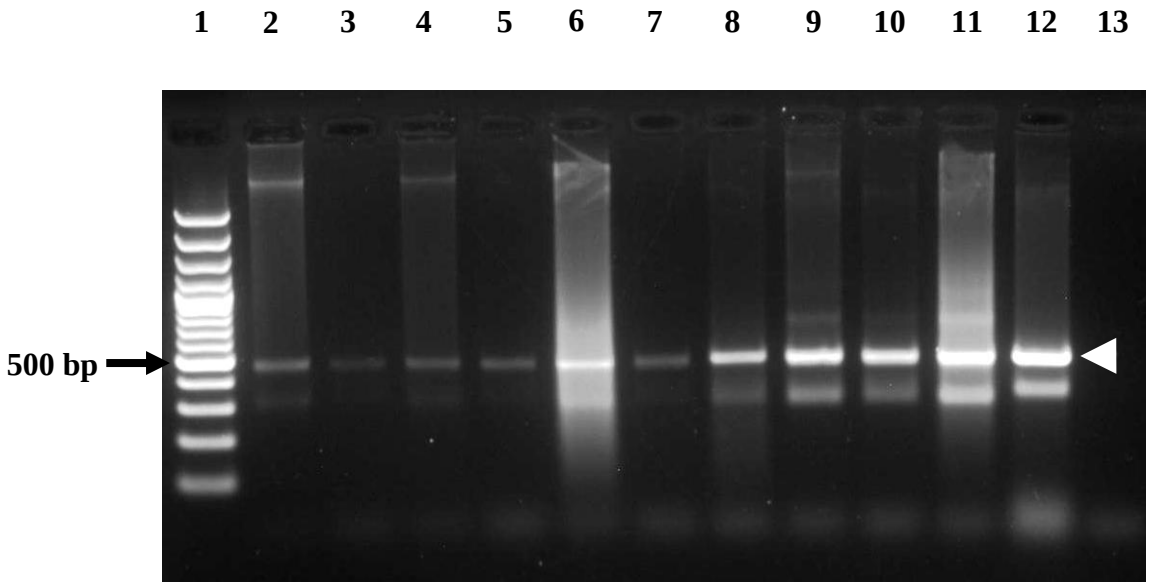


Figure 5.18 Bacterial 16S rDNA PCR of River Trent water samples. Lane 1:100 bp marker; Lane 2: NT09-01; Lane 3: NT09-02; Lane 4: NT09-03; Lane 5: NT09-04; Lane 6: NT09-05; Lane 7: NT09-06; Lane 8: NT09-07; Lane 9: NT09-08; Lane 10: NT09-09; Lane 11: NT09-10; Lane 12: Positive control (*E. coli* DNA); Lane 13: Negative control (nanopure water). White arrowhead shows the expected size (500 bp) of PCR product

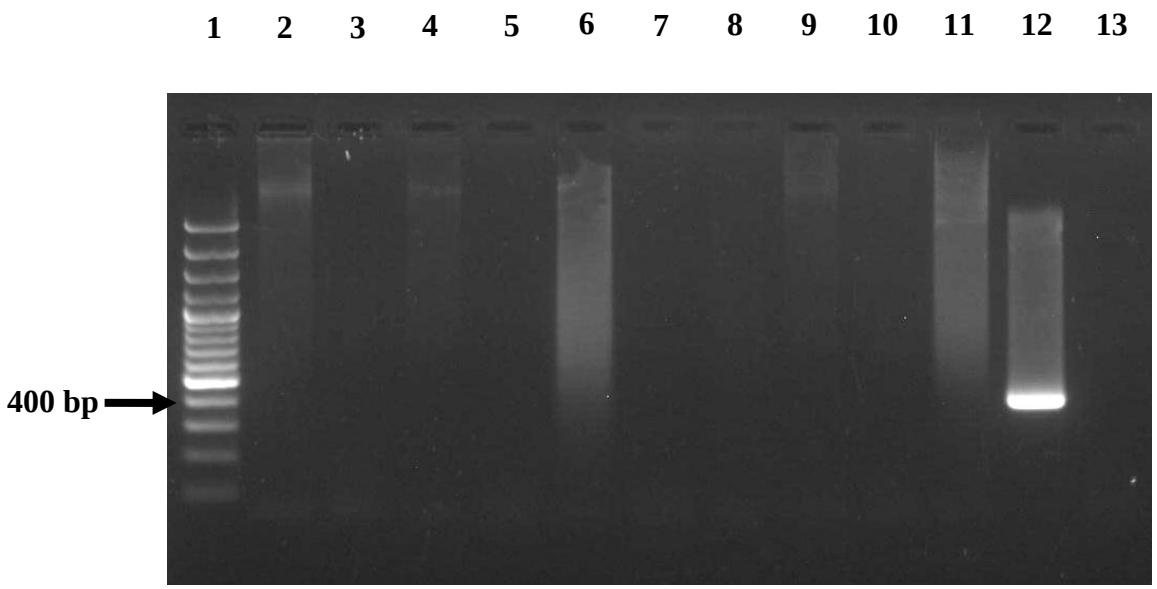


Figure 5.19 *B. mandrillaris* PCR using only Ext F/R primers with River Trent water samples. Lane 1:100 bp marker; Lane 2: NT09-01; Lane 3: NT09-02; Lane 4: NT09-03; Lane 5: NT09-04; Lane 6: NT09-05; Lane 7: NT09-06; Lane 8: NT09-07; Lane 9: NT09-08; Lane 10: NT09-09; Lane 11: NT09-10; Lane 12: Positive control (*B. mandrillaris* DNA); Lane 13: Negative control (nanopure water)

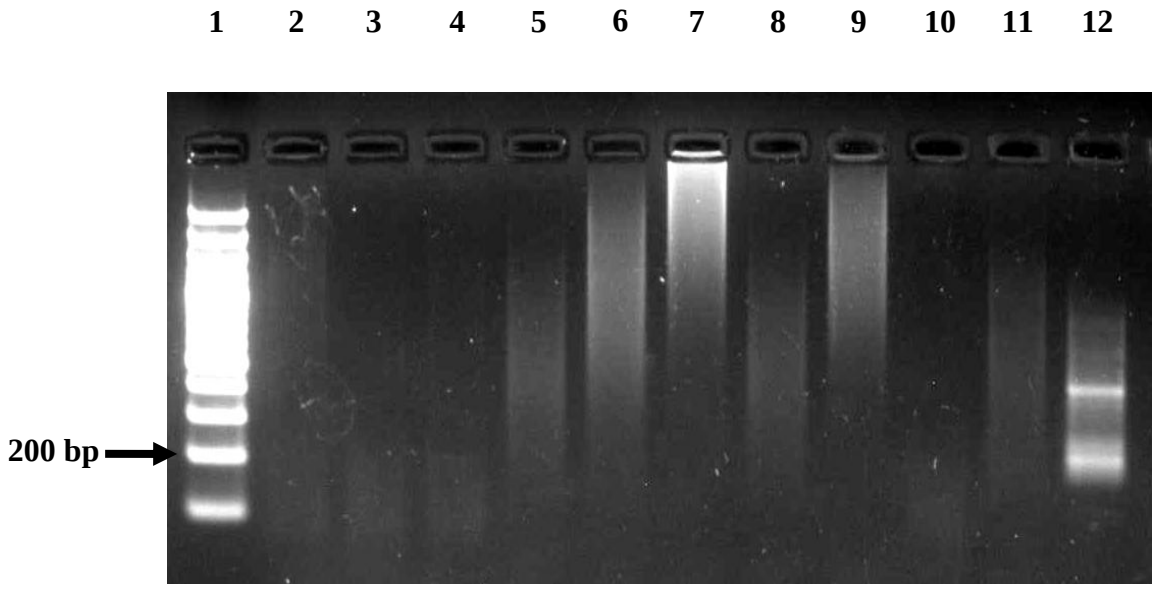


Figure 5.20 *B. mandrillaris* nested PCR with River Trent water samples. Lane 1:100 bp marker; Lane 2: NT09-01; Lane 3: NT09-02; Lane 4: NT09-03; Lane 5: NT09-04; Lane 6: NT09-05; Lane 7: NT09-06; Lane 8: NT09-07; Lane 9: NT09-08; Lane 10: NT09-09; Lane 11: NT09-10; Lane 12: Positive control (*B. mandrillaris* DNA)

5.2.2.3 Soil samples collected in the East Midlands, United Kingdom

As most *Balamuthia* infections are associated with gardening or agricultural work, a total of 16 soil samples were initially collected from gardens (7), flower bed/pots (6), road sides (2) and a park (1) in the East Midlands, United Kingdom (Table 5.3). High intensity and intact DNA fragment was seen on agarose gels as shown by representatives of the soil samples in Figure 5.21. The individual total DNA yields were between 7 to 20 µg/10 g and are listed in Table 5.3. All 16 samples showed positive PCR amplification using the bacterial 16S rDNA primers. As shown in Table 5.3 none were positive either with *B. mandrillaris* external primers alone or nested PCR. *B. mandrillaris* was not detected on NNA - *E. coli* plates incubated at both 32°C and 44°C. Other amoebae detected were *Acanthamoeba*, *Vahlkampfia*, *Hartmanella* and unknown amoeba (Table 5.3).

Table 5.3 Results of cultivation and PCR assays for East Midlands, United Kingdom soil samples

Sample	**Source**	**Total DNA yield (µg/10 g)**	**pH**	**Organisms cultured**		**Bacterial 16S rDNA PCR**	***B. mandrillaris* PCR**	
				32°C	**44°C**		**ExtF/R**	**Nested**
EM08-01	Flower bed	20	7	*Acanthamoeba*, *Vahlkampfia*	Unknown amoeba	+	Neg	Neg
EM08-02	Road side	16	7	*Acanthamoeba*	None	+	Neg	Neg
EM08-03	Road side	21	7	*Vahkampfia, Hartmanella*	None	+	Neg	Neg
EM08-04	Flower bed	13	7	*Acanthamoeba*	None	+	Neg	Neg
EM08-05	Flower bed	16	7	*Acanthamoeba*	None	+	Neg	Neg
EM08-06	Flower bed	15	7	*Acanthamoeba, Vahlkampfia*	*Vahlkampfia*	+	Neg	Neg
EM08-07	Flower bed	18	7	*Acanthamoeba*	None	+	Neg	Neg
EM08-08	Garden	8	7	*Acanthamoeba*	None	+	Neg	Neg
EM08-09	Garden	7	7	*Acanthamoeba*	None	+	Neg	Neg
EM08-10	Garden	11	7	*Acanthamoeba*	None	+	Neg	Neg
EM08-11	Flower pot	7	7	*Acanthamoeba*	None	+	Neg	Neg
EM08-12	Garden	11	7	*Acanthamoeba*	None	+	Neg	Neg
EM08-13	Garden	7	7	*Acanthamoeba*	None	+	Neg	Neg
EM08-14	Park	20	7	*Acanthamoeba*	None	+	Neg	Neg
EM08-15	Garden	12	6-7	*Acanthamoeba*, *Vahlkampfia*	None	+	Neg	Neg
EM08-16	Garden	13	6-7	*Acanthamoeba*, *Vahlkampfia*	None	+	Neg	Neg

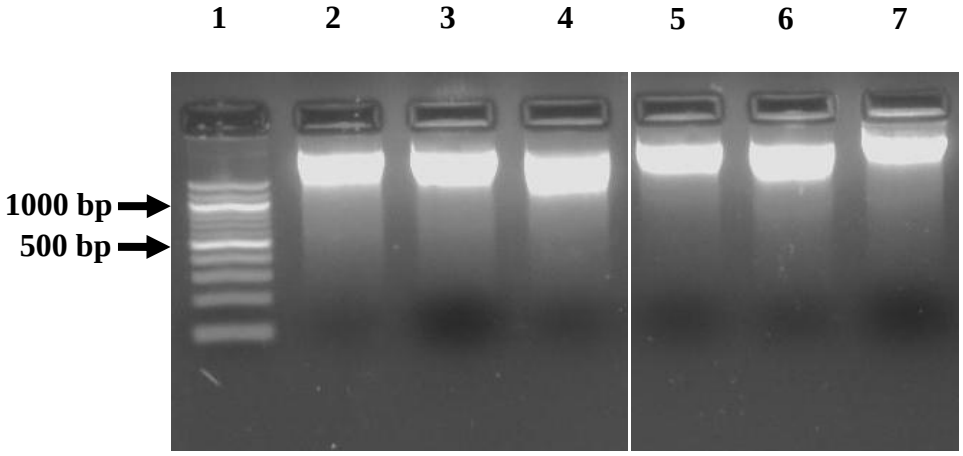


Figure 5.21 DNA yield after purification of East Midlands soils with ZR soil kit. Lane 1: 100 bp marker; Lane 2: EM08-08; Lane 3: EM08-09; Lane 4: EM08-10; Lane 5: EM08-11; Lane 6: EM08-12; Lane 7: EM08-13

5.2.2.4 Soil samples collected from the University of Reading farm, United Kingdom

A total of eighteen samples were collected from the University of Reading farm, United Kingdom. The samples were initially collected from nine different locations as shown in the soil map in Figure 5.22. Each sample was collected from two (0-10 cm and 10-20 cm) different soil depths and the details are shown in Table 5.4. Soil samples collected from locations 1 to 4 had the least organic content (1.8%). Moderate organic contents (3.8% to 14.7%) were obtained from locations 5 to 7 and the highest organic contents (22% to 24.9%) were from locations 8 and 9. All had a pH of 7 and visualisation on agarose gels showed similar DNA yield for soils collected from both depths (Figure 5.23). The DNA yields for soils from 0-10 cm and 10-20 cm depths were between 8 to 20 µg/10 g and 11 to 19 µg/10 g respectively (Table 5.4). All the eighteen soil samples showed positives results after the first round PCR with the bacterial 16S rDNA primers (Figures 5.24 and 5.25). *Balamuthia* DNA was not detected from either depths of soils

whether high organic (UR09-08, UR09-09, UR09-17 and UR09-18), moderate organic (UR09-05, UR09-06, UR09-07, UR09-14, UR09-15 and UR09-16) or poor organic content (UR09-01, UR09-02, UR09-03, UR09-04, UR09-10, UR09-11, UR09-12 and UR09-13), as shown in Figure 5.26 to Figure 5.29. Also *B. mandrillaris* was not found from whatever the land use for: No association was also seen between land use for winter wheat (UR09-01 and UR09-10), clover (UR09-02 and UR09-11), barley (UR09-03 and UR09-12), grassland (UR09-04, UR09-06, UR09-07, UR09-08, UR09-13, UR09-15, UR09-16 and UR09-17) or woodland (UR09-05, UR09-09, UR09-14 and UR09-18). Cultivation of samples of the soil suspension, on NNA-*E.coli* plates at 32°C, showed growth of *Acanthamoeba*, *Vahlkampfia*, *Naegleria*, *Tetrahymena* and worms. Growth of an unknown amoeba was seen at 44°C (Table 5.4).

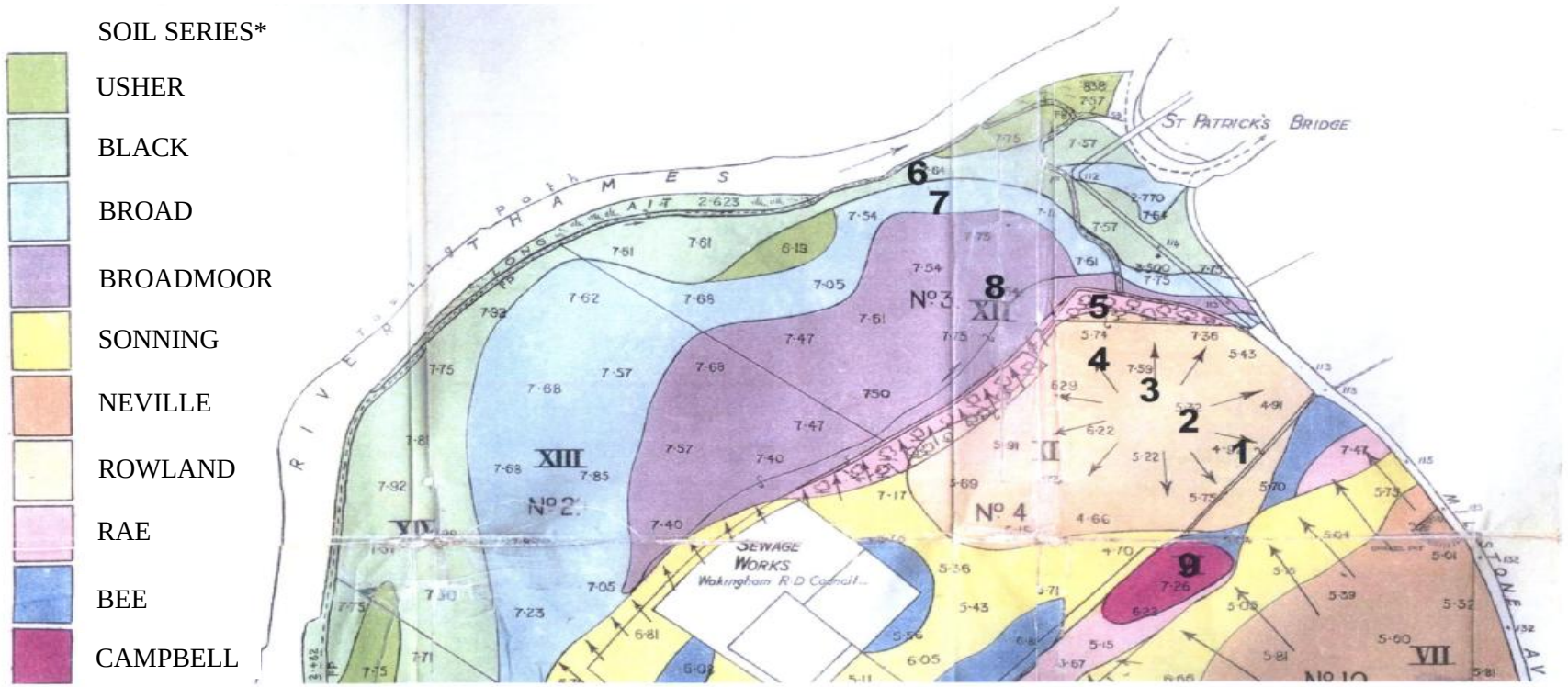


Figure 5.22 Soil map showing location of soil samples collected from the University of Reading farm, United Kingdom (Kay, 1936)

1: Samples UR09-01 and UR09-10 **4:** Samples UR09-04 and UR09-13 **7:** Samples UR09-07 and UR09-16

2: Samples UR09-02 and UR09-11 **5:** Samples UR09-05 and UR09-14 **8:** Samples UR09-08 and UR09-17

3: Samples UR09-03 and UR09-12 **6:** Samples UR09-06 and UR09-15 **9:** Samples UR09-09 and UR09-18

*Details of each soil series are described in Kay (1936)

Table 5.4 Details and cultivation results for soils collected from the University of Reading farm, United Kingdom

Sample	**Depth (cm)**	**Land use**	**Organic content (%)**	**Total DNA yield (µg/10 g)**	**pH**	**Organisms cultured**	
						32°C	**44°C**
UR09-01	0-10	Winter wheat	1.8	20	7	*Vahlkampfia*, *Acanthamoeba*	Unknown amoeba
UR09-02	0-10	Clover	1.8	19	7	*Acanthamoeba*	None
UR09-03	0-10	Barley	1.8	20	7	*Acanthamoeba*, *Tetrahymena*	None
UR09-04	0-10	Grassland	1.8	18	7	*Acanthamoeba*, *Vahlkampfia*, *Tetrahymena*	None
UR09-05	0-10	Woodland	3.8-8.1	11	7	*Acanthamoeba*, *Vahlkampfia*	Unknown amoeba
UR09-06	0-10	Grassland	11.5	12	7	*Acanthamoeba*, *Vahlkampfia*, worms	None
UR09-07	0-10	Grassland	14.7	17	7	*Acanthamoeba*, *Vahlkampfia*, *Naegleria*	None
UR09-08	0-10	Grassland	24.9	10	7	*Acanthamoeba*, *Vahlkampfia*, *Tetrahymena*, *Naegleria*	Unknown amoeba
UR09-09	0-10	Woodland	22	8	7	*Acanthamoeba*, *Naegleria*	None

Continued:

Sample	Depth (cm)	Land use	Organic content (%)	Total DNA yield (µg/10 g)	pH	Organisms cultured	
						32°C	44°C
UR09-10	10-20	Winter wheat	1.8	17	7	*Acanthamoeba, Naegleria*	Unknown amoeba
UR09-11	10-20	Clover	1.8	19	7	*Acanthamoeba, Vahlkampfia*	Unknown amoeba
UR09-12	10-20	Barley	1.8	14	7	*Acanthamoeba, Vahlkampfia*	None
UR09-13	10-20	Grassland	1.8	18	7	*Acanthamoeba*	None
UR09-14	10-20	Woodland	3.8-8.1	11	7	*Acanthamoeba, Vahlkampfia, Naegleria*	None
UR09-15	10-20	Grassland	11.5	13	7	*Acanthamoeba, Tetrahymena*	None
UR09-16	10-20	Grassland	14.7	19	7	*Acanthamoeba, Tetrahymena*	None
UR09-17	10-20	Grassland	24.9	16	7	*Acanthamoeba, Naegleria*	Unknown amoeba
UR09-18	10-20	Woodland	22	17	7	*Acanthamoeba, Naegleria*	None

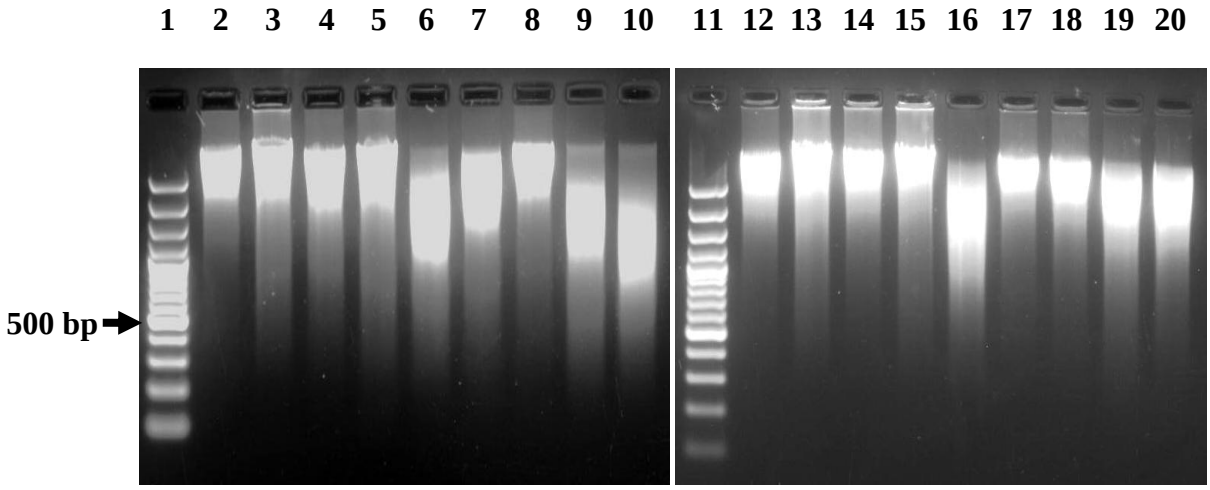


Figure 5.23 Agarose gels showing DNA yield after purification of University of Reading farm, United Kingdom soils with ZR soil kit. Lanes 1 and 11: 100 bp marker; Lane 2: UR09-01; Lane 3: UR09-02; Lane 4: UR09-03; Lane 5: UR09-04; Lane 6: UR09-05; Lane 7: UR09-06; Lane 8: UR09-07; Lane 9: UR09-08; Lane 10: UR09-09; Lane 12: UR09-10, Lane 13: UR09-11; Lane 14: UR09-12; Lane 15: UR09-13; Lane 16: UR09-14; Lane 17: UR09-15; Lane 18: UR09-16; Lane 19: UR09-17; Lane 20: UR09-18

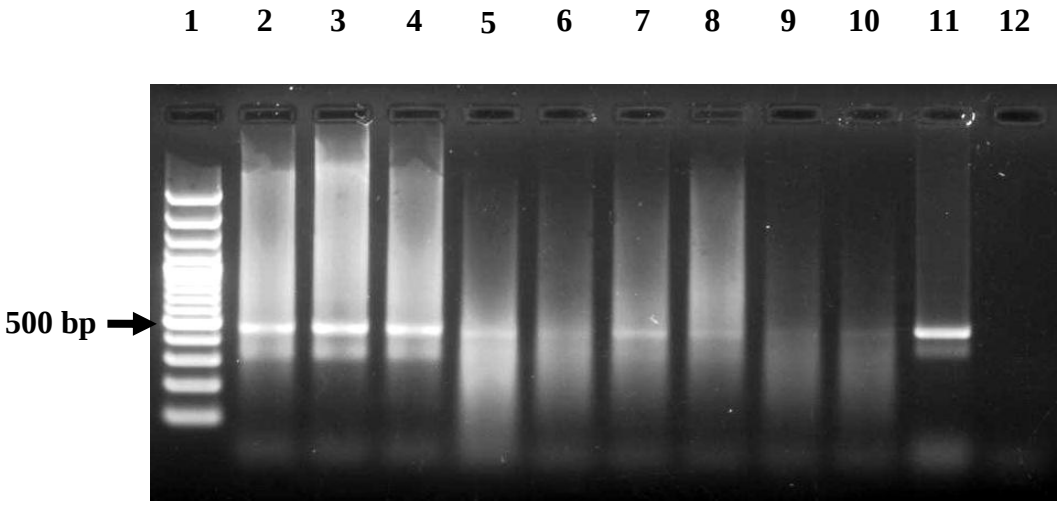


Figure 5.24 PCR amplification using bacterial 16S rDNA primers with soils (0-10 cm) from the University of Reading farm, United Kingdom. Lane 1: 100 bp marker; Lane 2: UR09-01; Lane 3: UR09-02; Lane 4: UR09-03; Lane 5: UR09-04; Lane 6: UR09-05; Lane 7: UR09-06; Lane 8: UR09-07; Lane 9: UR09-08; Lane 10: UR09-09; Lane 11: Positive control (*E. coli* DNA); Lane 12: Negative control (nanopure water)

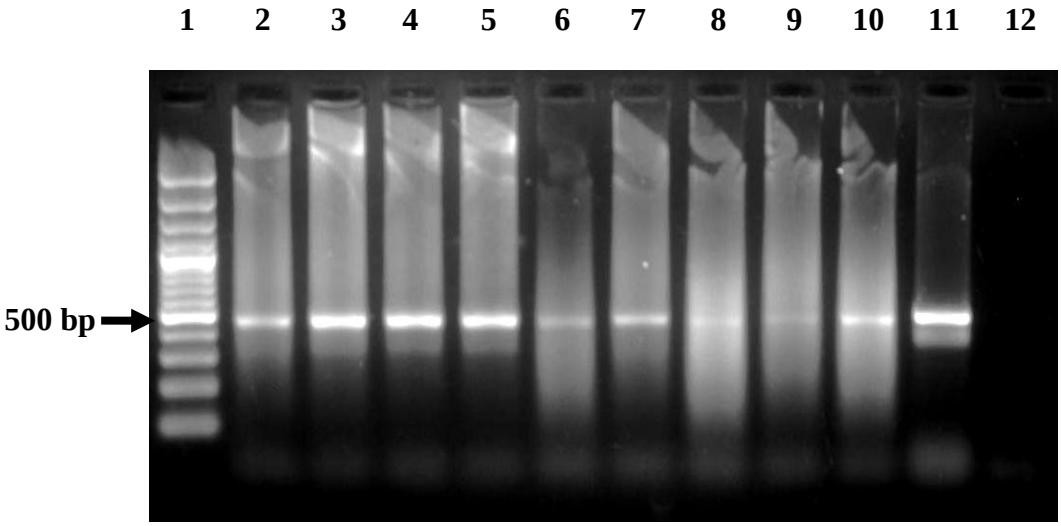


Figure 5.25 PCR amplification using bacterial 16S rDNA primers with soils (10-20 cm) from the University of Reading farm, United Kingdom. Lane 1: 100 bp marker; Lane 2: UR09-10; Lane 3: UR09-11; Lane 4: UR09-12; Lane 5: UR09-13; Lane 6: UR09-14; Lane 7: UR09-15; Lane 8: UR09-16; Lane 9: UR09-17; Lane 10: UR09-18; Lane 11: Positive control (*E. coli* DNA); Lane 12: Negative control (nanopure water)

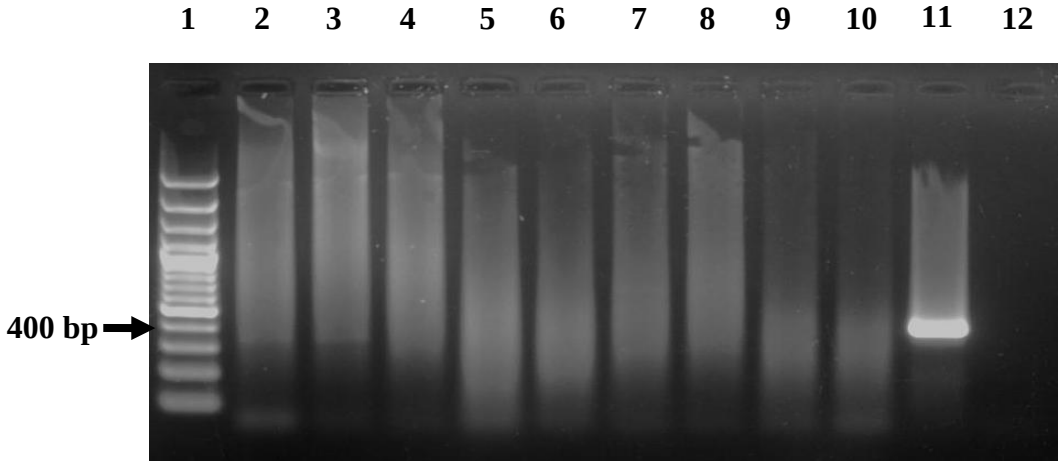


Figure 5.26 PCR amplification using *Balamuthia mandrillaris* external primers (ExtF/R) with soils (0-10 cm) from the University of Reading farm, United Kingdom. Lane 1: 100 bp marker; Lane 2: UR09-01; Lane 3: UR09-02; Lane 4: UR09-03; Lane 5: UR09-04; Lane 6: UR09-05; Lane 7: UR09-06; Lane 8: UR09-07; Lane 9: UR09-08; Lane 10: UR09-09; Lane 11: Positive control (*B. mandrillaris* DNA); Lane 12: Negative control (nanopure water)

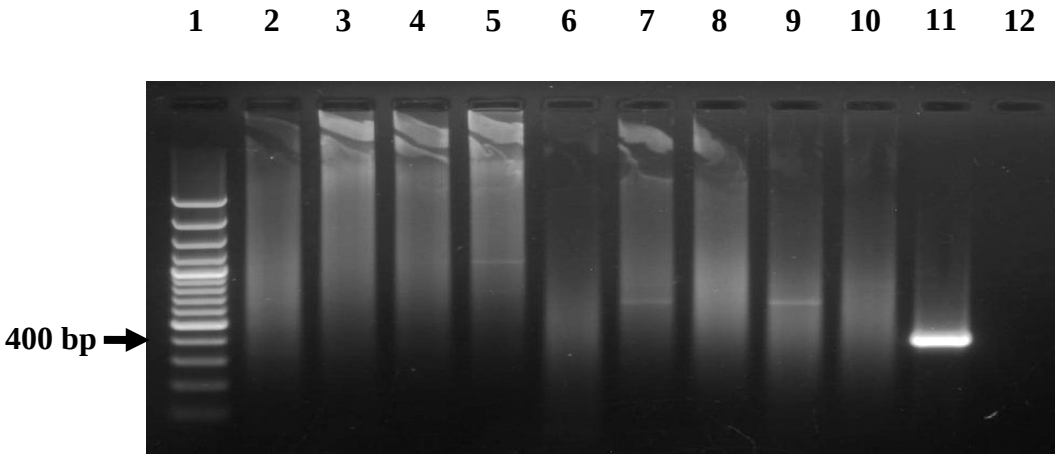


Figure 5.27 PCR amplification using *Balamuthia mandrillaris* external primers (ExtF/R) with soils (10-20 cm) from the University of Reading farm, United Kingdom. Lane 1: 100 bp marker; Lane 2: UR09-01; Lane 3: UR09-02; Lane 4: UR09-03; Lane 5: UR09-04; Lane 6: UR09-05; Lane 7: UR09-06; Lane 8: UR09-07; Lane 9: UR09-08; Lane 10: UR9-09; Lane 11: Positive control (*B. mandrillaris* DNA); Lane 12: Negative control (nanopure water)

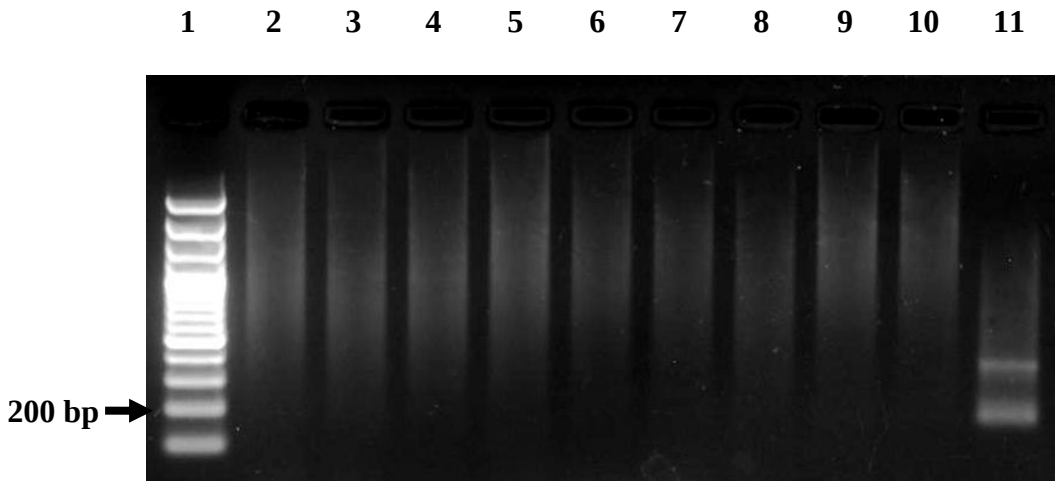


Figure 5.28 *Balamuthia mandrillaris* nested PCR with soils (0-10 cm) from the University of Reading farm, United Kingdom. Lane 1: 100 bp marker; Lane 2: UR09-01; Lane 3: UR09-02; Lane 4: UR09-03; Lane 5: UR09-04; Lane 6: UR09-05; Lane 7: UR09-06; Lane 8: UR09-07; Lane 9: UR09-08; Lane 10: UR09-09; Lane 11: Positive control (*B. mandrillaris* DNA)

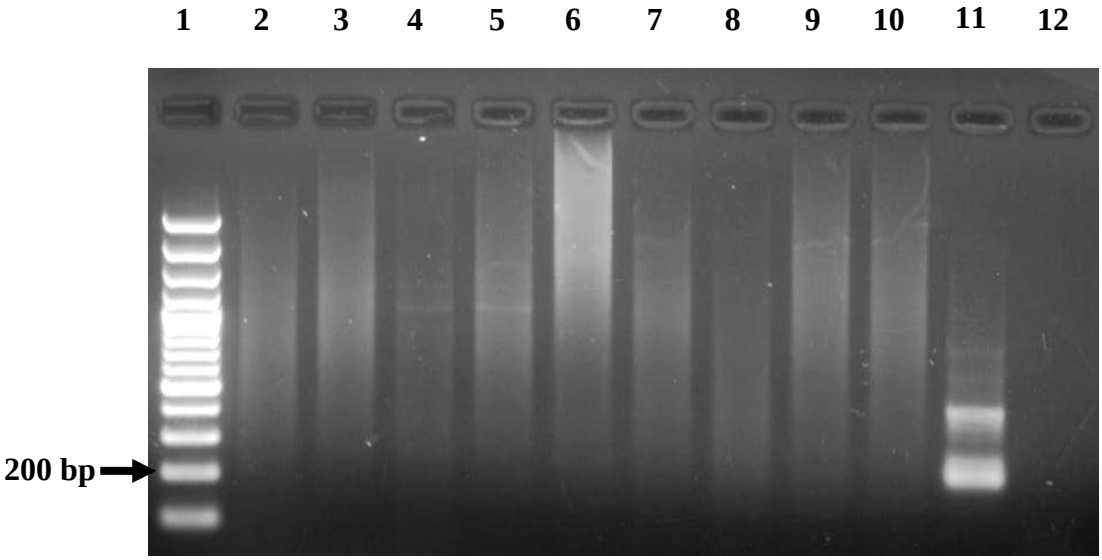


Figure 5.29 *Balamuthia mandrillaris* nested PCR with soils (10-20 cm) from the University of Reading farm, United Kingdom. Lane 1: 100 bp marker; Lane 2: UR09-10; Lane 3: UR09-11; Lane 4: UR09-12; Lane 5: UR09-13; Lane 6: UR09-14; Lane 7: UR09-15; Lane 8: UR09-16; Lane 9: UR09-17; Lane 10: UR09-18; Lane 11: Positive control (*B. mandrillaris* DNA); Lane 12: Negative control (nanopure water)

5.2.2.5 Soil samples collected in Southern California, USA

A total of 17 soil samples were collected from house gardens (15) and road sides (2) in Southern California, USA and the details are shown in Table 5.5. All samples had a pH of 7 as measured by pH 1-11 indicator paper (Whatman, UK). The DNA yield for each of the samples is shown in Figure 5.30. Three of the soil samples (SC09-01, SC09-04 and SC09-06, Lanes 2, 5, and 7) showed an almost invisible band and quantitative measurement showed DNA yields of 1 µg/10 g, 2 µg/10 g and 1 µg/10 g respectively (Table 5.5). All but two were positives for bacterial DNA during the first round PCR with bacterial 16S rDNA primers with very faint band seen for SC09-04 and SC09-06 (Figure 5.31). As shown in Figure 5.32, both samples (CS10-01, Lane 1) and CS10-02, Lane 2) showed positive results upon reamplification. Four samples (SC09-01, SC09-

09, SC09-10 and SC09-11) showed a very faint band with the external *Balamuthia* primers (Figure 5.33, Lanes 2, 10, 11 and 12). Figure 5.34 shows 16 (SC09-01, SC09-02, SC09-03, SC09-04, SC09-05, SC09-06, SC09-07, SC09-08, SC09-09, SC09-10, SC09-11, SC09-12, SC09-14, SC09-15, SC09-16 and SC09-17) positive samples detected by the nested PCR. Nine (SC09-02, SC09-03, SC09-04, SC09-05, SC09-07, SC09-09, SC09-10, SC09-11 and SC09-12) of the positive samples were sent for sequencing and all showed 100% identity to the reference *B. mandrillaris* strains (GenBank accession numbers; AF477019, AF477020, AF477021, AF477022 and AF019071) deposited in the GenBank. *B. mandrillaris* was not detected after incubation at 32°C and 44°C (Table 5.5). Other amoebae detected were *Acanthamoeba*, *Vahlkampfia*, *Rhizamoeba* and an unknown amoeba (Table 5.5).

Table 5.5 Details and cultivation results for soils collected from Southern California

Sample	**Source**	**pH**	**Total DNA yield µg/10 g**	**Organisms cultured**	
				32°C	**44°C**
SC09-01	Garden	7	1	*Acanthamoeba*, *Vahlkampfia*	*Acanthamoeba*, *Vahlkampfia*, unknown amoeba
SC09-02	Garden	7	16	*Acanthamoeba*, *Vahlkampfia*, unidentified fungus	*Acanthamoeba*, *Vahlkampfia*, unidentified fungus
SC09-03	Garden	7	8	*Acanthamoeba*, *Vahlkampfia*	*Acanthamoeba*, *Vahlkampfia*
SC09-04	Garden	7	2	*Acanthamoeba*, *Vahlkampfia*, unidentified fungus	*Acanthamoeba*, *Vahlkampfia*, unidentified fungus
SC09-05	Garden	7	18	*Acanthamoeba*, *Vahlkampfia*	*Acanthamoeba*
SC09-06	Garden	7	1	*Acanthamoeba*, *Vahlkampfia*	*Acanthamoeba*, *Vahlkampfia*
SC09-07	Garden	7	17	*Acanthamoeba*, *Vahlkampfia*	*Vahlkampfia*
SC09-08	Road side	7	20	*Acanthamoeba*, *Vahlkampfia*	*Acanthamoeba*, unknown amoeba
SC09-09	Garden	7	8	*Acanthamoeba*, *Vahlkampfia*	*Acanthamoeba*

Continued:

Sample	Source	pH	Total DNA yield µg/10 g	Organisms cultured	
				32°C	44°C
SC09-10	Garden	7	18	*Acanthamoeba*, *Vahlkampfia*	*Acanthamoeba*, *Vahlkampfia*, unknown amoeba
SC09-11	Garden	7	13	*Acanthamoeba*, *Rhizamoeba*,	*Acanthamoeba*
SC09-12	Garden	7	20	*Acanthamoeba*, *Vahlkampfia*	*Vahlkampfia*
SC10-01	Garden	7	18	*Acanthamoeba*, *Vahlkampfia* worm	*Acanthamoeba*
SC10-02	Road side	7	8	*Acanthamoeba*, *Vahlkampfia*	*Vahlkampfia*
SC10-03	Garden	7	6	*Acanthamoeba*, *Vahlkampfia*	*Acanthamoeba*
SC10-04	Garden	7	11	*Acanthamoeba*, *Vahlkampfia*, worm	*Acanthamoeba*, *Vahkampfia*
SC10-05	Garden	7	5	*Acanthamoeba*, *Vahlkampfia*	*Acanthamoeba*, worm

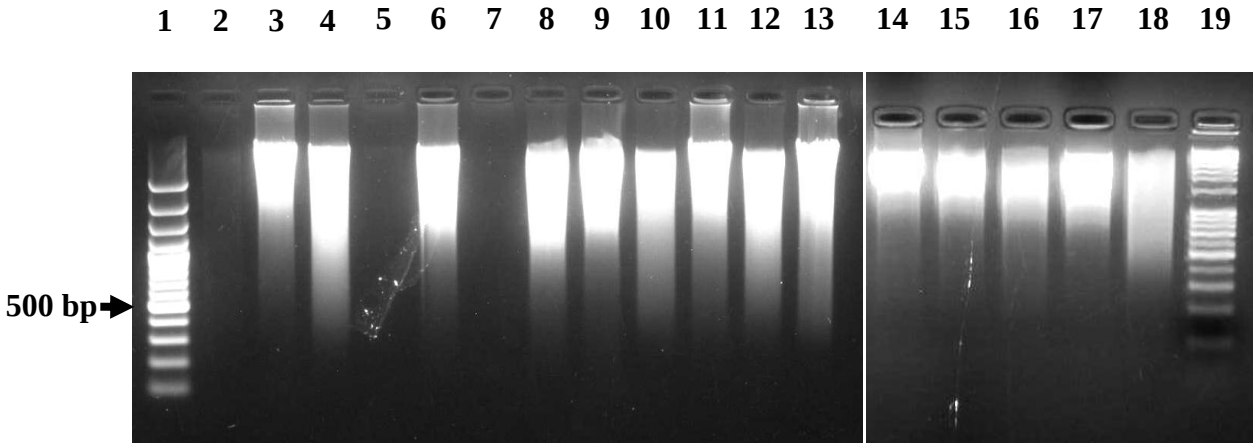


Figure 5.30 Agarose gels showing DNA yield after purification of soils from Southern California, USA with ZR soil kit. Lanes 1: 100 bp marker; Lane 2: SC09-01; Lane 3: SC09-02; Lane 4: SC09-03; Lane 5: SC09-04; Lane 6: SC09-05; Lane 7: SC09-06; Lane 8: SC09-07; Lane 9: SC09-08; Lane 10: SC09-09; Lane 11: SC09-10, Lane 12: SC09-11; Lane 13: SC09-12; Lane 14: SC10-01; Lane 15: SC10-02; Lane 16: SC10-03; Lane 17: SC10-04; Lane 18: SC10-05; Lane 19: 1000 bp marker

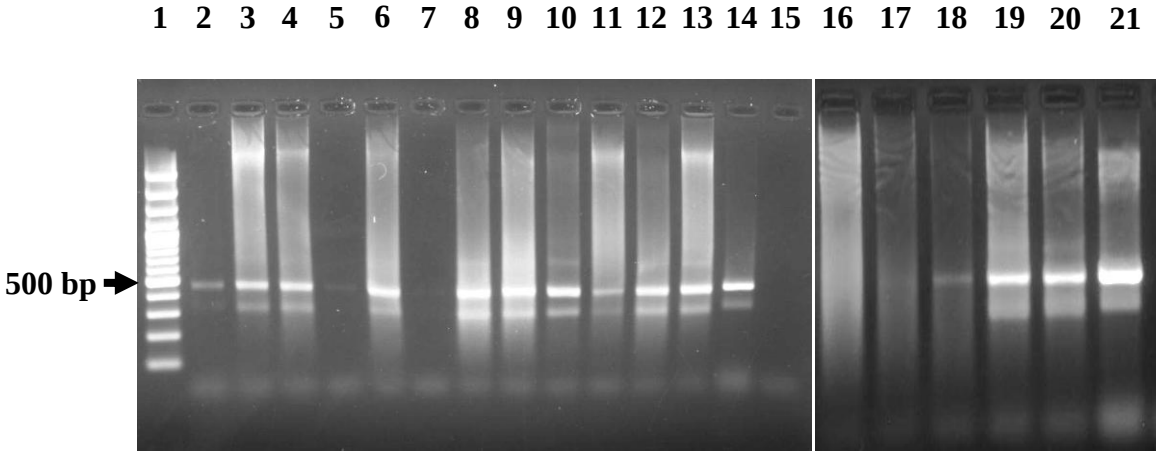


Figure 5.31 PCR amplification using bacterial 16S rDNA primers with soils collected from Southern California, USA. Lanes 1: 100 bp marker; Lane 2: SC09-01; Lane 3: SC09-02; Lane 4: SC09-03; Lane 5: SC09-04; Lane 6: SC09-05; Lane 7: SC09-06; Lane 8: SC09-07; Lane 9: SC09-08; Lane 10: SC09-09; Lane 11: SC09-10, Lane 12: SC09-11; Lane 13: SC09-12; Lane 14: Positive control (*E. coli* DNA); Lane 15: Negative control (nanopure water); Lane 16: SC10-01; Lane 17: SC10-02; Lane 18: SC10-03; Lane 19: SC10-04; Lane 20: SC10-05; Lane 21: Positive control (*E. coli* DNA)

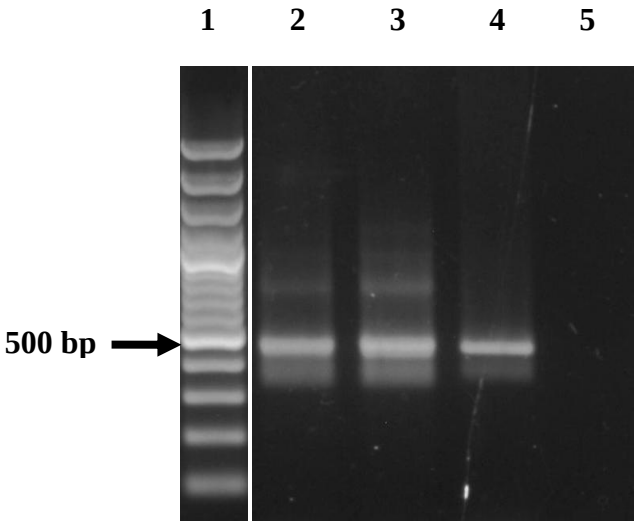


Figure 5.32 DNA reamplification of samples SC10-01 and SC10-02 with bacterial 16S rDNA primers. Lane 1: 100 bp marker; Lane 2: SC10-01; Lane 3: SC10-02; Lane 4; Positive control (*E. coli* DNA); Lane 5: Negative control (nanopure water)

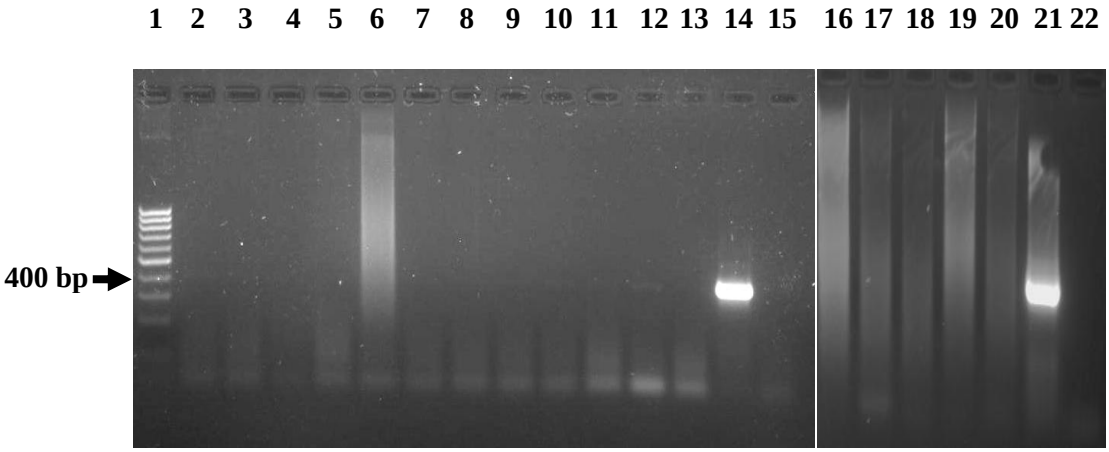


Figure 5.33 PCR amplification using *B. mandrillaris* external (ExtF/R) primers with soils collected from Southern California, USA. Lanes 1: 100 bp marker; Lane 2: SC09-01; Lane 3: SC09-02; Lane 4: SC09-03; Lane 5: SC09-04; Lane 6: SC09-05; Lane 7: SC09-06; Lane 8: SC09-07; Lane 9: SC09-08; Lane 10: SC09-09; Lane 11: SC09-10, Lane 12: SC09-11; Lane 13: SC09-12; Lane 14: Positive control (*B. mandrillaris* DNA); Lane 15: Negative control (nanopure water); Lane 16: SC10-01; Lane 17: SC10-02; Lane 18: SC10-03; Lane 19: SC10-04; Lane 20: SC10-05; Lane 21: Positive control (*B. mandrillaris* DNA); Lane 22: Negative control (nanopure water)

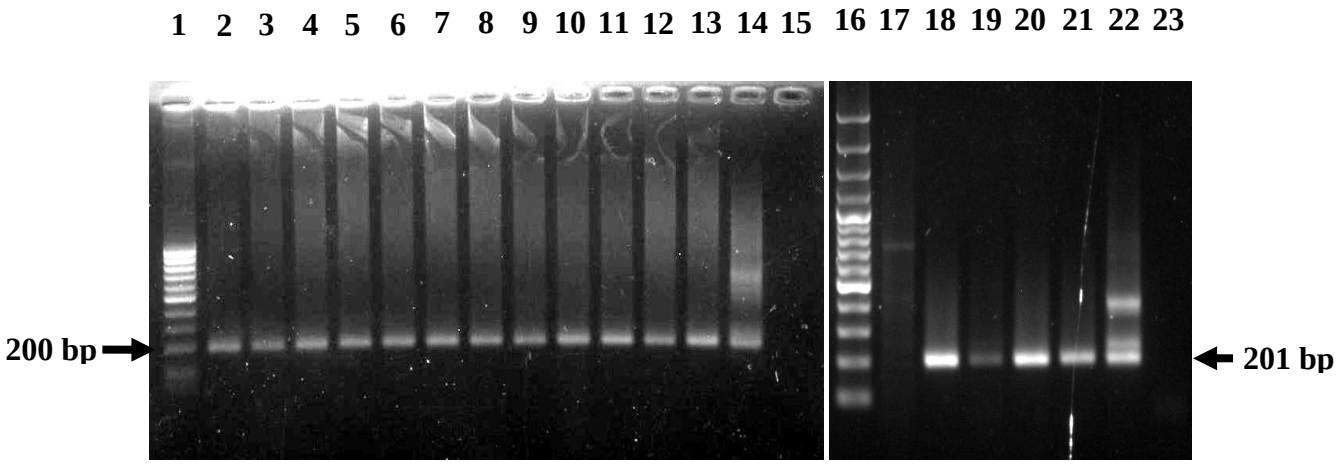


Figure 5.34 *B. mandrillaris* nested PCR with soils collected from Southern California, USA. Lanes 1: 100 bp marker; Lane 2: SC09-01; Lane 3: SC09-02; Lane 4: SC09-03; Lane 5: SC09-04; Lane 6: SC09-05; Lane 7: SC09-06; Lane 8: SC09-07; Lane 9: SC09-08; Lane 10: SC09-09; Lane 11: SC09-10, Lane 12: SC09-11; Lane 13: SC09-12; Lane 14: Positive control (*B. mandrillaris* DNA); Lane 15: Negative control (nanopure water); Lane 16: 100 bp marker; Lane 17: SC10-01; Lane 18: SC10-02; Lane 19: SC10-03; Lane 20: SC10-04; Lane 21: SC10-05; Lane 22: Positive control (*B. mandrillaris* DNA); Lane 23: Negative control (nanopure water)

5.2.2.6 Soil samples collected in the Republic of South Africa

Samples SA08-01 and SA08-02 were collected from Kruger Park and Cape Town, South Africa. The DNA yield and 16S rDNA bacterial PCR results are shown in Figure 5.35 A and B. Amplification using only the *B. mandrillaris* external primers yielded negative results (Figure 5.36A). However, both samples (SA08-01, SA08-02) were positive by the *B. mandrillaris* nested PCR with the expected PCR product of 201 bp (Figure 5.36B). Cultivation of both soils at 32°C and 44°C resulted in growth of *Acanthamoeba*.

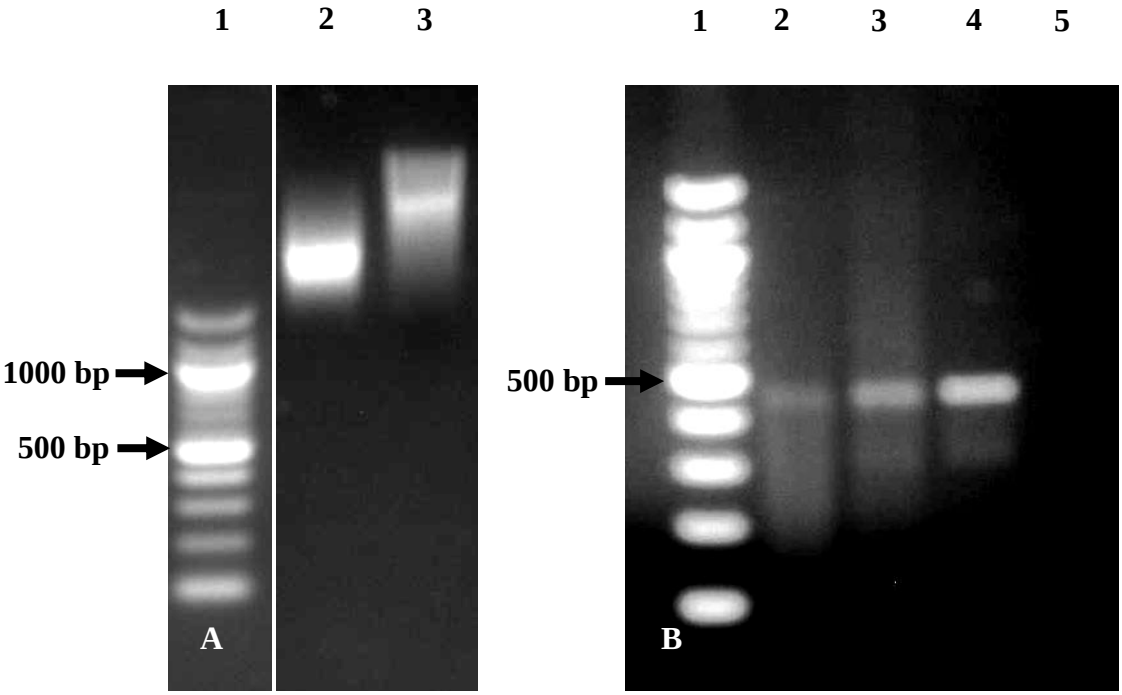


Figure 5.35 DNA yield after purification using ZR soil kit (A) and bacterial 16S rDNA PCR (B) with South Africa soils. A. Lane 1: 100 bp marker; Lane 2: SA08-01; Lane 3: SA08-02. B. Lane 1: 100 bp marker; Lane 2: SA08-01; Lane 3: SA08-02; Lane 4: Positive control (*E. coli* DNA); Lane 5: Negative control (nanopure water)

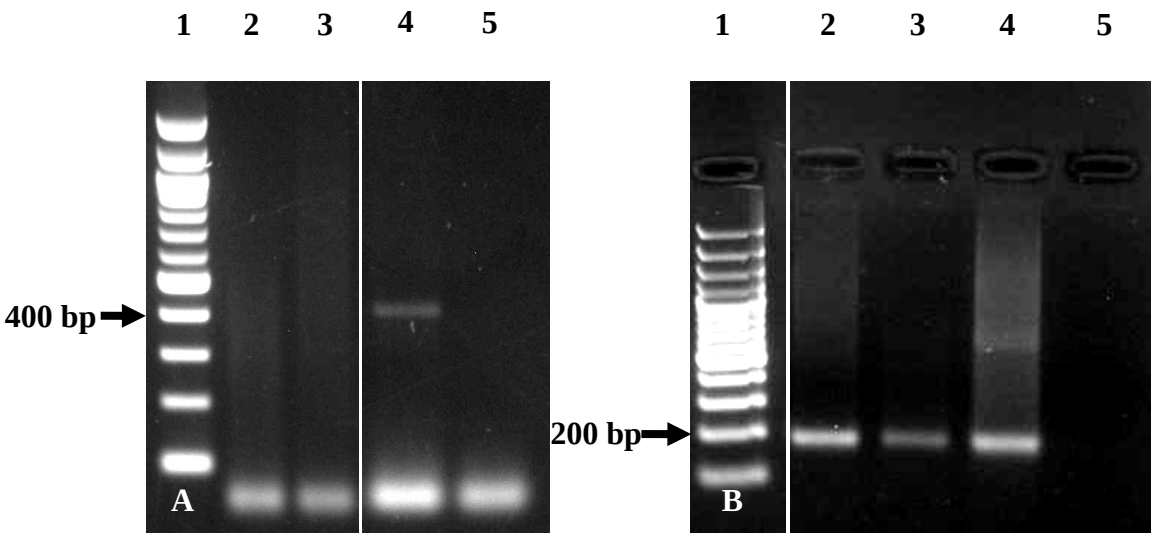


Figure 5.36 PCR amplification using *B. mandrillaris* PCR external primers (**A**) and nested primers (**B**) with South Africa soils. **A**. Lane 1: 100 bp marker; Lane 2: SA08-01; Lane 3: SA08-02; Lane 4: Positive control (*B. mandrillaris* DNA); Lane 5: Negative control (nanopurewater). **B**. Lane 1: 100 bp marker; Lane 2: SA08-01; Lane 3: SA08-02; Lane 4: Positive control (*B. mandrillaris* DNA); Lane 5: Negative control (nanopure water)

5.2.2.7 Soil samples collected in Italy

Six soil samples were obtained from Italy and details of each sample is compiled in Table 5.6. The DNA yield of the soils, measured by spectrophotometry, ranging from 6 µg/10 g to 25 µg/10 g, while on agarose gels, high intensity and intact DNA bands were shown for all of the soils except IT09-06 (Lane 7), as shown in Figure 5.37. Only 4 (IT09-01, IT09-03, IT09-04 and IT09-05) out of 6 samples showed positive DNA amplification during the first round of bacterial 16S rDNA PCR (Figure 5.38). DNA reamplification of both the negative samples using the same bacterial 16S rDNA primers showed PCR products with the expected size of 500 bp as shown in Figure 5.39. Detection of *B. mandrillaris* DNA, using only the external primers, yielded negative results (Figure 1.41). Reamplification of the PCR product by the nested

primers showed a very strong band with size less than 200 bp for sample IT09-01 (Figure 5.41, Lane 2) and a very faint band with the expected size (201 bp) from samples IT09-03 (Figure 5.41, Lane 4). The PCR products from both samples were sent for sequencing. Results showed no sequence similarity of IT09-01 to any sequences in the GenBank. There was insufficient DNA for sequencing of the sample IT09-03. Cultivation of soil suspensions at 32°C showed growth of *Acanthamoeba*, *Naegleria*, *Tetrahymena*, *Vahlkampfia* and unknown amoebae. All of the soil samples, except IT09-06, showed no growth of amoebae at 44°C (Table 5.6).

Table 5.6 Details and cultivation results for Italian soils

Sample	**Source**	**pH**	**Total DNA yield (µg/10 g)**	**Organisms cultured**	
				32°C	**44°C**
IT09-01	Under olive tree	7	25	*Naegleria*, *Tetrahymena*, *Acanthamoeba* unknown amoeba	None
IT09-02	Under trees in forest	7	8	*Naegleria*, *Vahlkampfia*	None
IT09-03	Area which is rich in clay and fossils	7	25	*Vahlkampfia*, unknown amoeba	None
IT09-04	Grassland	7	18	*Acanthamoeba*, unknown amoeba	None
IT09-05	Wheat field	7	14	*Acanthamoeba*, unknown amoeba	None
IT09-06	Under fig tree	7	6	*Tetrahymena*	*Vahlkampfia*, unknown amoeba

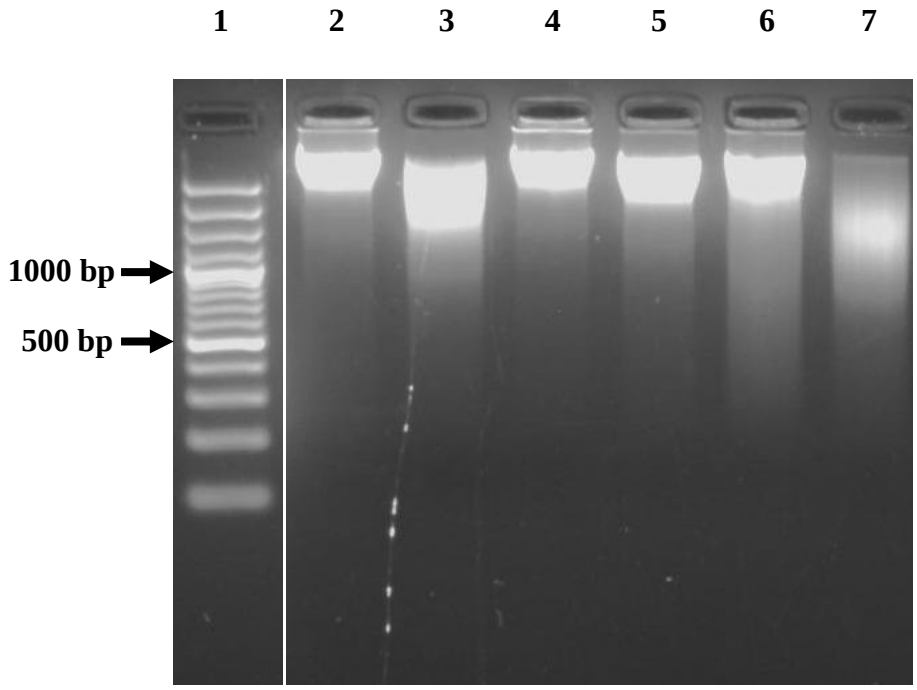


Figure 5.37 DNA yield after purification of Italian soil with ZR soil kit. Lane 1: 100 bp marker; Lane 2: IT09-01; Lane 3: IT09-02; Lane 4: IT09-03; Lane 5: IT09-04; Lane 6: IT09-05; Lane 7: IT09-06

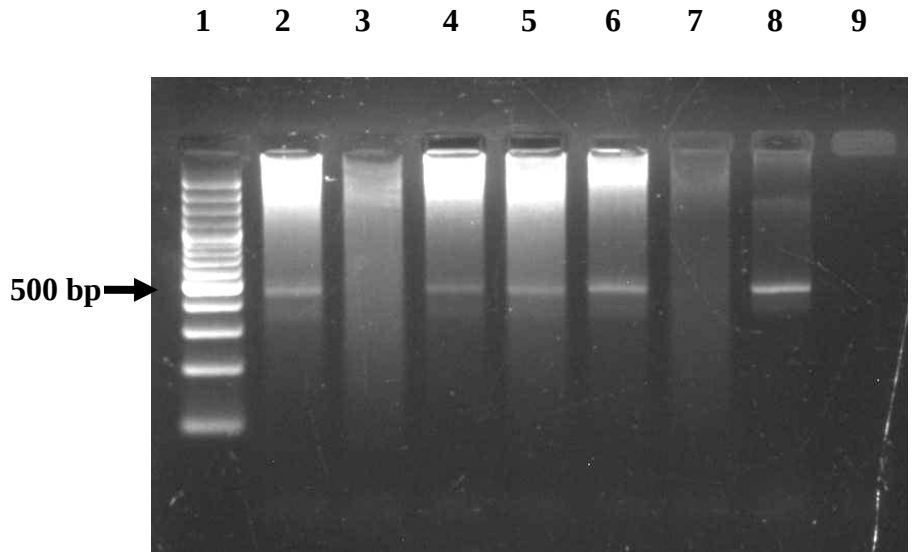


Figure 5.38 PCR amplification using bacterial 16S rDNA primers with Italian soils. Lane: 1: 100 bp marker; Lane 2: IT09-01; Lane 3: IT09-02; Lane 4: IT09-03; Lane 5: IT09-04; Lane 6: IT09-05; Lane 7: IT09-06; Lane 8: Positive control (*E. coli* DNA); Lane 9: Negative control (nanopure water)

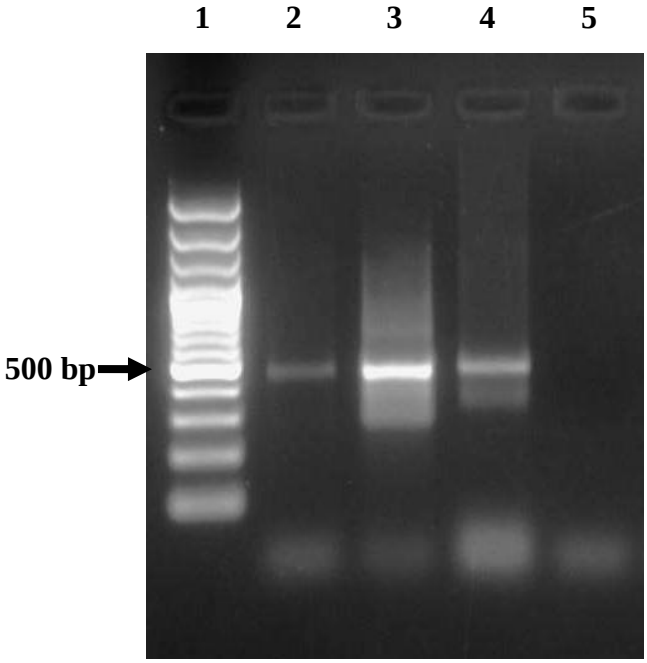


Figure 5.39 DNA reamplification of samples IT09-02 and IT09-06 with bacterial 16S rDNA primers. Lane 1: 100 bp marker; Lane 2: IT09-02; Lane 3: IT09-06; Lane 4: Positive control (*E. coli* DNA); Lane 5: Negative control (nanopure water)

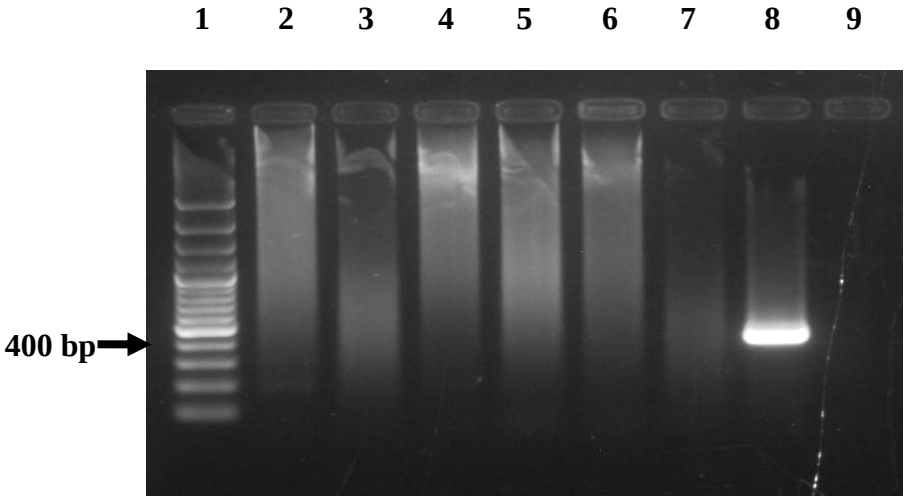


Figure 5.40 PCR amplification using *B. mandrillaris* external (ExtF/R) primers with soils collected from Italy. Lane: 1: 100 bp marker; Lane 2: IT09-01; Lane 3: IT09-02; Lane 4: IT09-03; Lane 5: IT09-04; Lane 6: IT09-05; Lane 7: IT09-06; Lane 8: Positive control (*B. mandrillaris* DNA); Lane 9: Negative control (nanopure water)

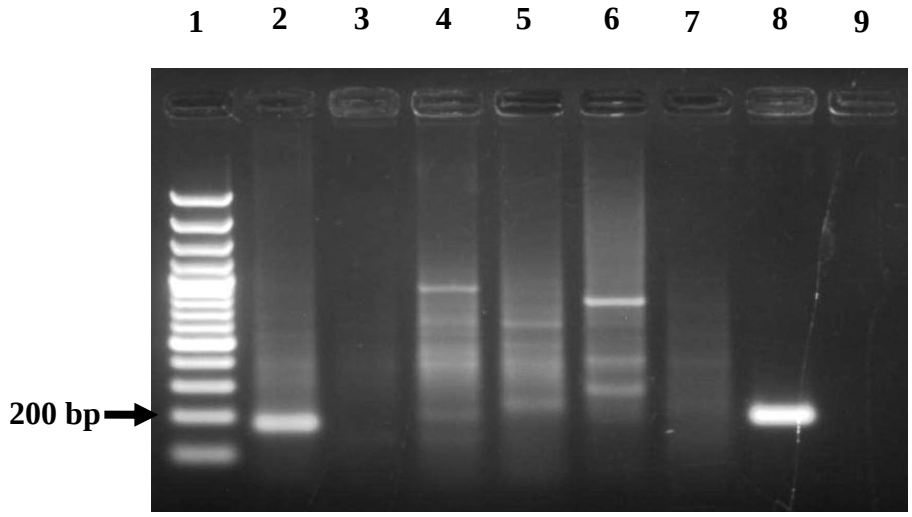


Figure 5.41 *B. mandrillaris* nested PCR with soils collected from Italy. Lane: 1: 100 bp marker; Lane 2: IT09-01; Lane 3: IT09-02; Lane 4: IT09-03; Lane 5: IT09-04; Lane 6: IT09-05; Lane 7: IT09-06; Lane 8: Positive control (*B. mandrillaris* DNA); Lane 9: Negative control (nanopure water)

5.2.2.8 Soil samples collected in Portugal

Eight soil samples were collected from Loule, Portugal. The source, pH and total DNA yield for each of the sample is shown in Table 5.7. Three of the samples (PT09-03, PT09-04 and PT09-05 did not show visible DNA on agarose gel electrophoresis (Figure 5.42, Lanes 4 to 6). Quantitative measurement of these three samples showed low DNA yield (0.4 µg/10 g, 2 µg/10 g and 1 µg/10 g respectively) compared to the other five samples (Table 5.7). Only two of the samples (PT09-07 and PT09-08) showed positive bacterial DNA amplification after the first round PCR (Figure 5.43) while the other six (PT09-01, PT09-02, PT09-03, PT09-04, PT09-05 and PT09-06) were only positive upon reamplification using the same bacterial 16S rDNA primers (Figure 5.44). None of the samples were positive for *B. mandrillaris* DNA, using the external primers alone (Figure 5.45). *B. mandrillaris* nested PCR (Figure 5.46) yielded one positive sample

(PT09-07) and DNA sequencing (Appendix 2) showed 100% identity to *B. mandrillaris* DNA sequences deposited in the GenBank. *B. mandrillaris* was not detected by culture at 32°C and 44°C. *Acanthamoeba* was the most frequently detected amoeba at 32°C. At 44°C, all samples did not show growth of amoeba, except PT09-07 with growth of *Acanthamoeba* and an unknown amoeba (Table 5.7).

Table 5.7 Details and cultivation results for soils collected from Portugal

Sample	**Source**	**pH**	**Total DNA yield (µg/10 g)**	**Organisms cultured**	
				32°C	**44°C**
PT09-01	Field with crops (*Ceratonia siliqua* and *Avena sativa*)	7	9	*Tetrahymena*, *Acanthamoeba*, *Vahlkampfia*	None
PT09-02	Road-side	7	17	*Tetrahymena*, *Acanthamoeba*, *Vahlkampfia*	None
PT09-03	Field with different types of flowers (*Cistus* sp.) and shrubs	7	0.4	*Acanthamoeba*, *Vahlkampfia*	None
PT09-04	Field with crops and flowers (*Ceratonia siliqua*, *Olea europaea* and *Ditrichia viscosa*)	7	2	*Acanthamoeba*	None
PT09-05	Field with trees *(Quercus rotundifolia)* and flowers (*Cistus* sp.)	7	1	*Acanthamoeba*	None
PT09-06	Land near a river	7	8	*Tetrahymena*, *Vahlkampfia*	None
PT09-07	Field with trees (*Quercus suber*) and flowers (*Cistus* sp.)	7	3	*Acanthamoeba*	*Acanthamoeba*, unknown amoeba
PT09-08	Land with compost	7	8	*Acanthamoeba*	None

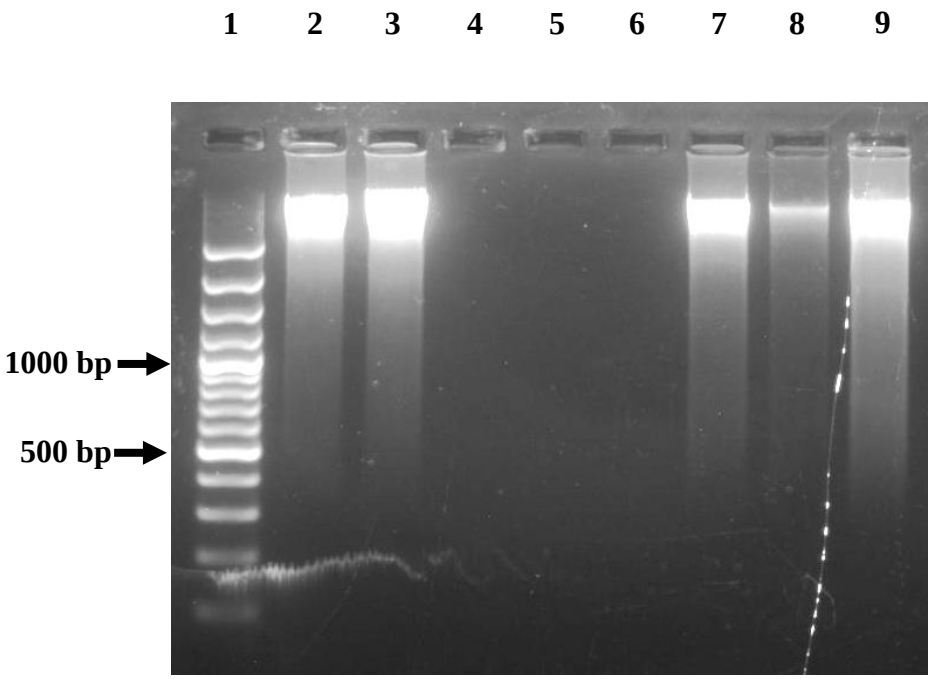


Figure 5.42 DNA yield after purification of soils from Portugal with ZR soil kit. Lane 1: 100 bp marker; Lane 2: PT09-01; Lane 3: PT09-02; Lane 4: PT09-03; Lane 5: PT09-04; Lane 6: PT09-05; Lane 7: PT09-06; Lane 8: PT09-07; Lane 9: PT09-08

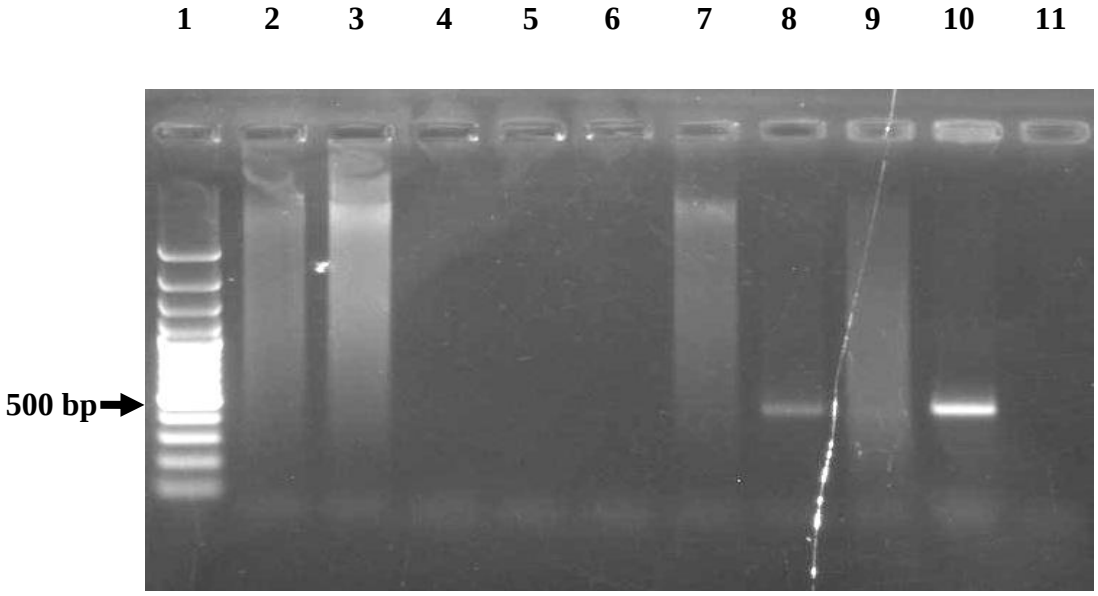


Figure 5.43 PCR amplification using bacterial 16S rDNA primers with soils for Portugal. Lane: 1: 100 bp marker; Lane 2: PT09-01; Lane 3: PT09-02; Lane 4: PT09-03; Lane 5: PT09-04; Lane 6: PT09-05; Lane 7: PT09-06; Lane 8: PT09-07; Lane 9: PT09-08; Lane 10: Positive control (*E. coli* DNA); Lane 11: Negative control (nanopure water)

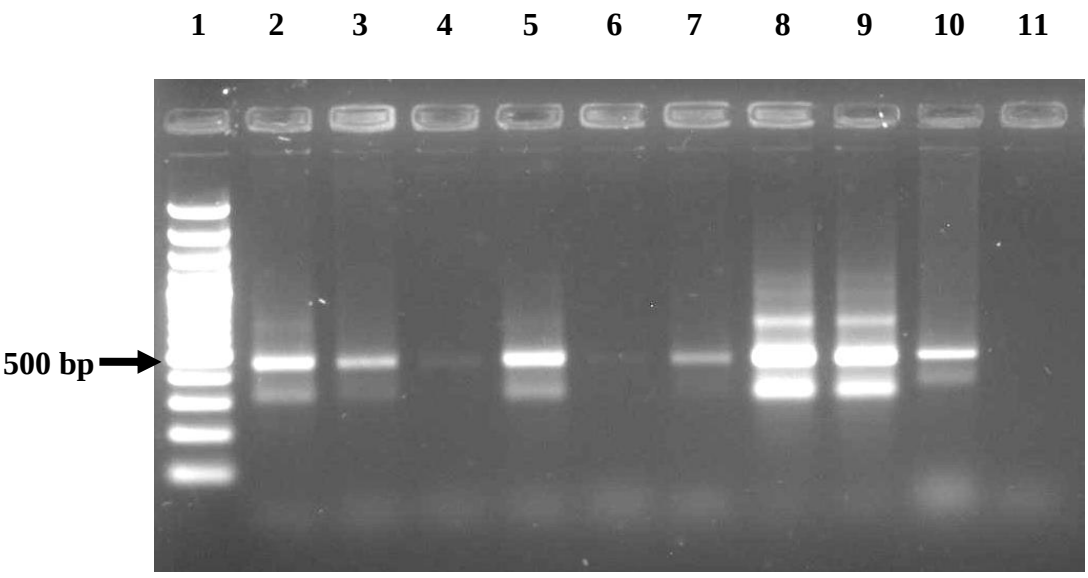


Figure 5.44 DNA reamplification of Portugal soils with bacterial 16S rDNA primers. Lane: 1: 100 bp marker; Lane 2: PT09-01; Lane 3: PT09-02; Lane 4: PT09-03; Lane 5: PT09-04; Lane 6: PT09-05; Lane 7: PT09-06; Lane 8: PT09-07; Lane 9: PT09-08; Lane 10: Positive control (*E. coli* DNA); Lane 11: Negative control (nanopure water)

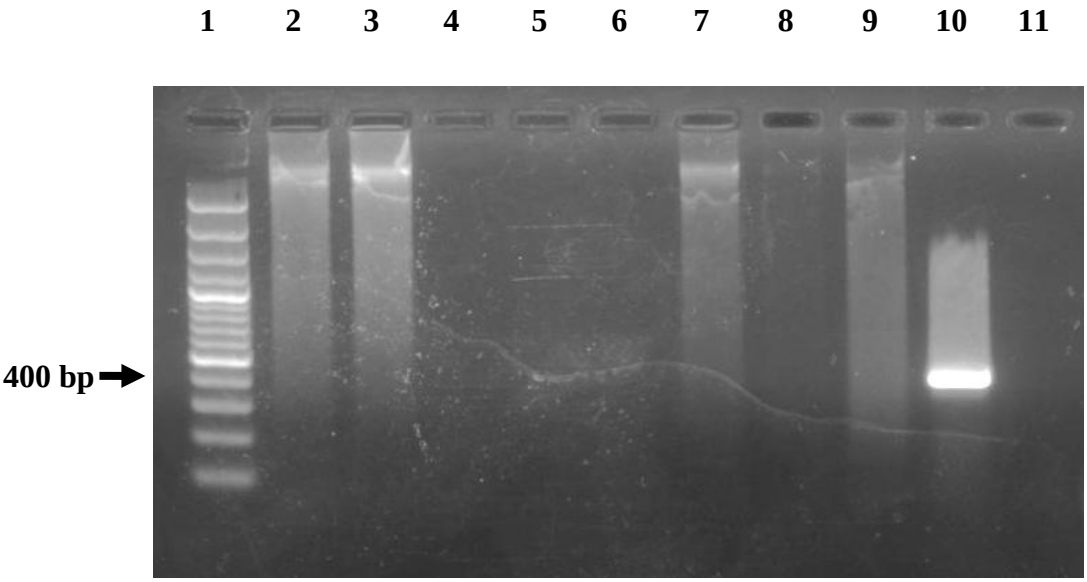


Figure 5.45 PCR amplification using *B. mandrillaris* external primers (ExtF/R) with soils collected from Portugal. Lane: 1: 100 bp marker; Lane 2: PT09-01; Lane 3: PT09-02; Lane 4: PT09-03; Lane 5: PT09-04; Lane 6: PT09-05; Lane 7: PT09-06; Lane 8: PT09-07; Lane 9: PT09-08; Lane 10: Positive control (*B. mandrillaris* DNA); Lane 11: Negative control (nanopure water)

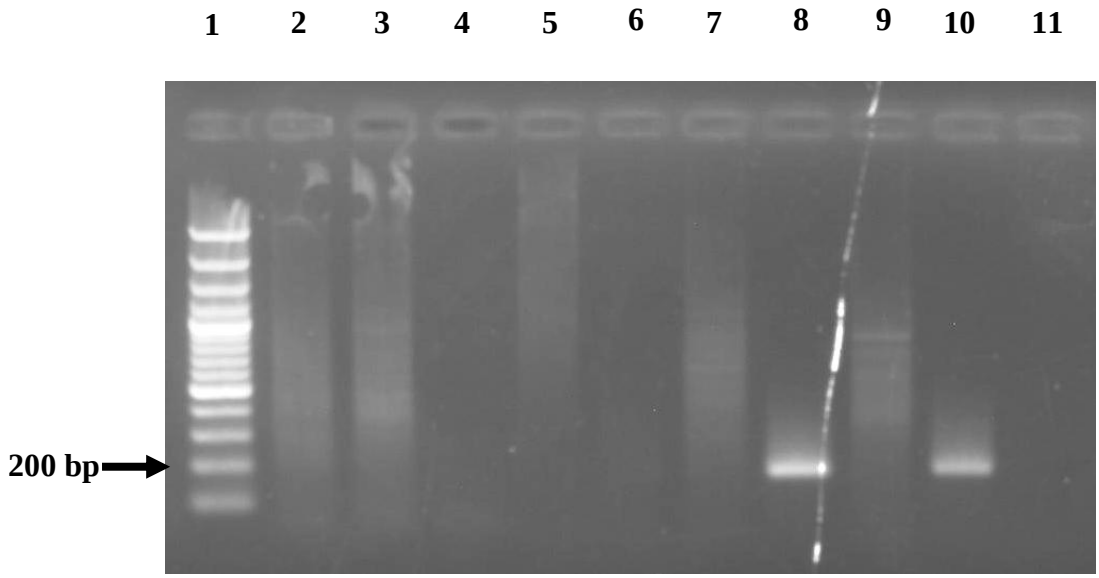


Figure 5.46 *B. mandrillaris* nested PCR with soils collected from Portugal. Lane: 1: 100 bp marker; Lane 2: PT09-01; Lane 3: PT09-02; Lane 4: PT09-03; Lane 5: PT09-04; Lane 6: PT09-05; Lane 7: PT09-06; Lane 8: PT09-07; Lane 9: PT09-08; Lane 10: Positive control (*B. mandrillaris* DNA); Lane 11: Negative control (nanopure water)

5.2.3 Confirmation of PCR positive samples using published primers

A total of sixteen South Californian soil samples which were detected to be positive using the developed nested PCR were tested further using two sets of published primers designed from the *B. mandrillaris* nuclear 18S rDNA and mitochondrial 16S rDNA sequences (see Table 2.3 for details). As shown in Figure 5.47, ten samples (SC09-02, SC09-03, SC09-05, SC09-07, SC09-08, SC09-09, SC09-10, SC09-11, SC09-12 and SC10-05) were found to be positive using the nuclear 18S rDNA primers with the expected PCR product of 171 bp. PCR using the mitochondrial 16S rDNA primers resulted in multiple and inconsistently sized PCR products, with seven samples (SC09-01, SC09-02, SC09-03, SC09-05, SC09-10, SC09-11and SC10-02) showing a band at the expected size of 1075 bp (Figure 5.48).

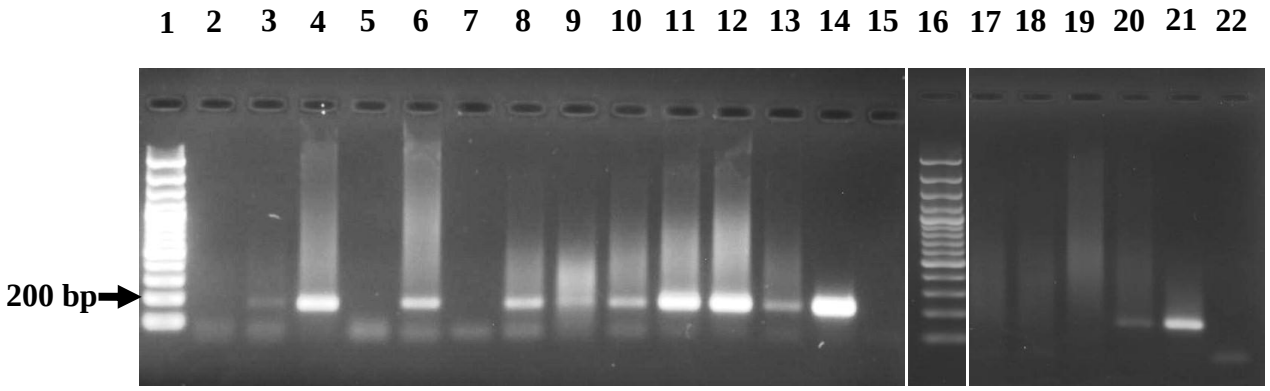


Figure 5.47 PCR amplification using *B. mandrillaris* nuclear 18S rDNA primers for South Californian soils. Lanes 1 and 16: 100 bp marker; Lane 2: SC09-01; Lane 3:SC09-02; Lane 4: SC09-03; Lane 5: SC09-04; Lane 6: SC09-05; Lane 7: SC09-06; Lane 8: SC09-07; Lane 9: SC09-08; Lane 10: SC09-09; Lane 11: SC09-10; Lane 12: SC09-11; Lane 13: SC09-12; Lane 14: Positive control (*B. mandrillaris* DNA); Lane 15: Negative control (nanopure water); Lane 17: SC10-02; Lane 18: SC10-03; Lane 19: SC10-04; Lane 20: SC10-05; Lane 21: Positive control (*B. mandrillaris* DNA); Lane 22: Negative control (nanopure water)

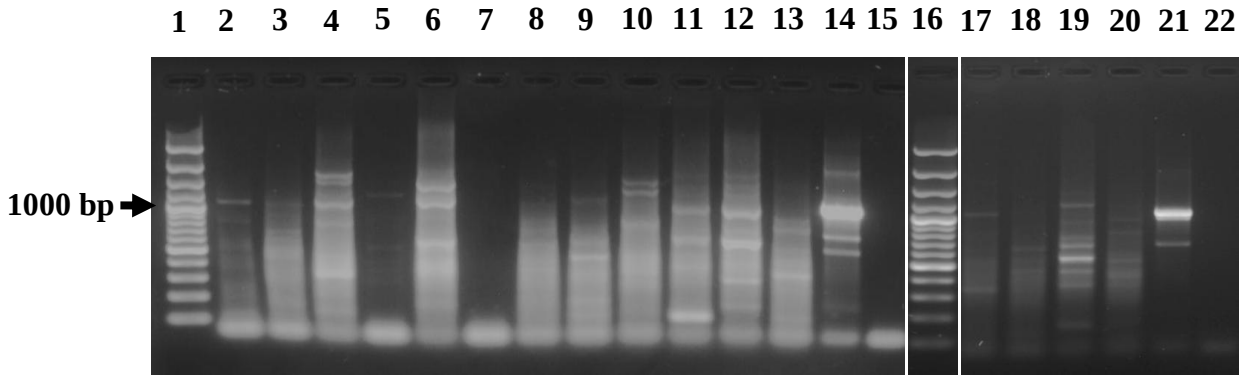


Figure 5.48 PCR amplification using *B. mandrillaris* mitochondrial 16S rDNA primers for South Californian soils. Lanes 1 and 16: 100 bp marker; Lane 2: SC09-01; Lane 3:SC09-02; Lane 4: SC09-03; Lane 5: SC09-04; Lane 6: SC09-05; Lane 7: SC09-06; Lane 8: SC09-07; Lane 9: SC09-08; Lane 10: SC09-09; Lane 11: SC09-10; Lane 12: SC09-11; Lane 13: SC09-12; Lane 14: Positive control (*B. mandrillaris* DNA); Lane 15: Negative control (nanopure water); Lane 17: SC10-02; Lane 18: SC10-03; Lane 19: SC10-04; Lane 20: SC10-05; Lane 21: Positive control (*B. mandrillaris* DNA); Lane 22: Negative control (nanopure water)

5.2.4 Detection of *Naegleria fowleri* from environmental samples using the developed DNA extraction method and one-step nested PCR

5.2.4.1 Detection of *N. fowleri* from water samples collected in France and the United Kingdom

An in-house one-step nested PCR assay was utilised for the detection of *N. fowleri* DNA from environmental samples. PCR amplification using either OP4F/R or IP4F/R against the *N. fowleri* plasmid DNA (see Table 2.1 and Section 2.20) produced a prominent band at 767 bp (Lane 2) and 506 bp (Lane 4) respectively (Figure 5.49). When a one-step nested PCR was performed using the positive control, two distinct bands which correspond to the outer and internal primers were seen (Figure 5.50).

Following PCR screening using bacterial 16S rDNA primers (Sections 5.2.2.1 and 5.2.2.2), a total of 10 water samples from River Trent, Nottinghamshire, United Kingdom and 109 from River Tarn, France were determined to be suitable for PCR and were tested further with the *N. fowleri* nested PCR. DNA amplification with these water samples yielded 18 positives out of 119 samples tested (Table 5.8). All the positives were from the River Tarn, France. Sixteen of these water samples (FW08-27, FW08-28, FW08-67, FW08-68, FW08-69, FW08-70, FW08-71, FW08-72, FW08-73, FW08-74, FW08-75, FW08-82, FW09-07, FW09-08, FW09-11 and FW09-34) were from clear water and one each was water with sediment (FW08-19) and water with algae (FW08-76). Representatives of the positive results are shown in lanes 2 and 3 of Figure 5.51. DNA sequencing of 4 positive samples (FW08-27, FW08-28, FW08-68 and FW08-72, Appendix 2) showed 99% homology to the DNA sequence of the cloned *N. fowleri* used to design the one-step nested PCR. The nested PCR results with the River Trent, Nottinghamshire water samples are shown in Figure 5.52. Cultivation of the French water samples at 44°C revealed growth of *Vahlkampfia*, *Hartmanella*, *Cashia*, *Vanella*,

Platyamoeba, *Acanthamoeba* and an unknown amoeba (Table 5.8). No *N. fowleri* or flagellates were found.

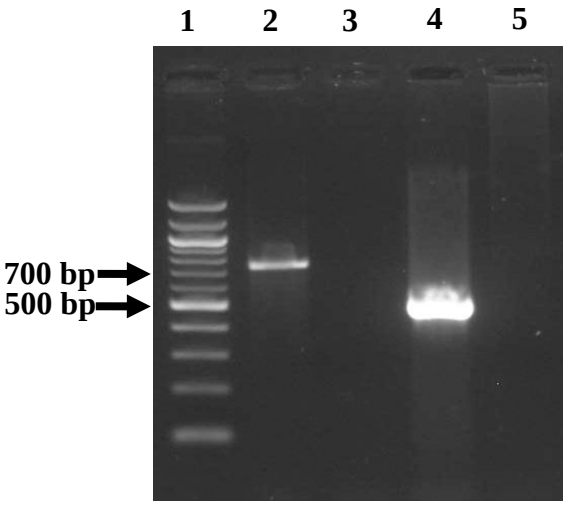


Figure 5.49 PCR amplification of *N. fowleri* plasmid DNA using OP4F/R and IP4F/R primers. Lane 1: 100 bp marker; Lane 2: *N. fowleri* + OP4F/R (767 bp); Lane 3: Negative control; Lane 4: *N. fowleri* + IP4F/R (506 bp); Lane 5: Negative control (nanopure water)

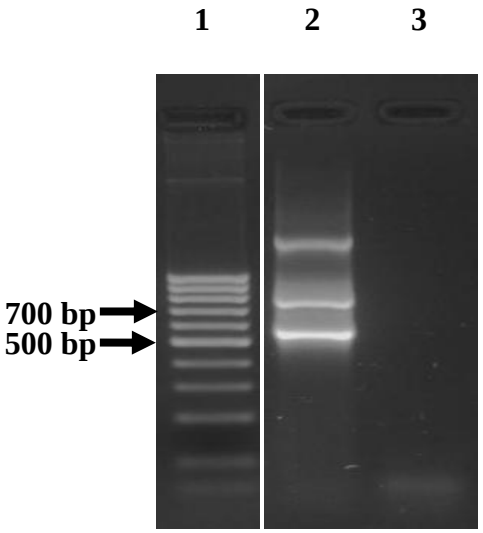


Figure 5.50 Amplification of *N. fowleri* plasmid DNA by single-step nested PCR. Lane 1: 100 bp marker; Lane 2: *N. fowleri* + OP4F/R + IP4F/R; Lane 3: Negative control (nanopure water)

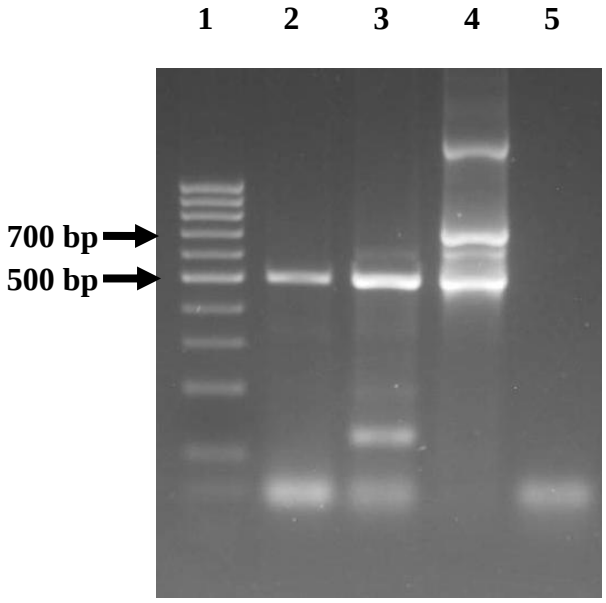


Figure 5.51 Detection of *N. fowleri* DNA with River Tarn, France water samples by nested PCR. Lane 1: 100 bp marker; Lane 2: FW08-75; Lane 3: FW08-76; Lane 4: Positive control (*N. fowleri* plasmid DNA); Lane 5: Negative control (nanopure water)

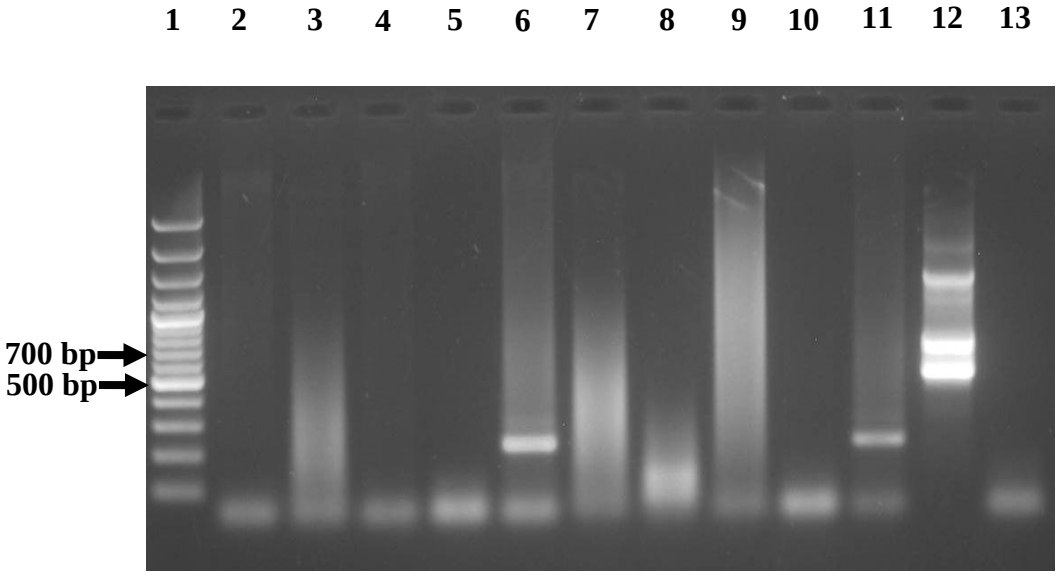


Figure 5.52 *N. fowleri* nested PCR for River Trent, Nottinghamshire water samples. Lane 1: 100 bp marker; Lane 2: NT09-01; Lane 3: NT09-02; Lane 4: NT09-03; Lane 5: NT09-04; Lane 6: NT09-05; Lane 7: NT09-06; Lane 8: NT09-07; Lane 9: NT09-08; Lane 10: NT09-09; Lane 11: NT09-10; Lane 12: Positive control (*N. fowleri* plasmid DNA); Lane 13: Negative control (nanopure water)

Table 5.8 Detection of *N. fowleri* from River Tarn, South West France water samples

Sample	**Organisms cultured at 44°C**		**Nested PCR**
	N. fowleri	**Others**	***N. fowleri***
FW08-19	None	*Hartmanella*	+
FW08-20	None	*Hartmanella*	Neg
FW08-21	None	None	Neg
FW08-22	None	None	Neg
FW08-23	None	*Vahlkampfia*	Neg
FW08-24	None	*Vahlkampfia*	Neg
FW08-25	None	None	Neg
FW08-26	None	*Hartmanella*	Neg
FW08-27	None	None	+
FW08-28	None	None	+
FW08-29	None	None	Neg
FW08-30	None	None	Neg
FW08-31	None	*Hartmanella*	Neg
FW08-32	None	None	Neg
FW08-33	None	None	Neg
FW08-34	None	None	Neg
FW08-35	None	None	Neg
FW08-36	None	None	Neg
FW08-37	None	None	Neg
FW08-38	None	None	Neg
FW08-39	None	None	Neg
FW08-40	None	None	Neg
FW08-41	None	*Hartmanella*	Neg
FW08-42	None	None	Neg
FW08-43	None	*Hartmanella*	Neg
FW08-44	None	*Hartmanella*	Neg
FW08-45	None	*Hartmanella*	Neg
FW08-46	None	*Cashia*	Neg

Continued

Sample	Organisms cultured at 44°C		Nested PCR
	N. fowleri	Others	*N. fowleri*
FW08-47	None	*Hartmanella*	Neg
FW08-48	None	None	Neg
FW08-49	None	*Vanella*	Neg
FW08-50	None	*Hartmanella*	Neg
FW08-51	None	None	Neg
FW08-52	None	*Hartmanella*	Neg
FW08-53	None	*Hartmanella*	Neg
FW08-54	None	*Hartmanella*	Neg
FW08-55	None	*Hartmanella, Acanthamoeba*	Neg
FW08-56	None	None	Neg
FW08-57	None	*Hartmanella*	Neg
FW08-58	None	*Hartmanella*	Neg
FW08-59	None	*Hartmanella, Vahlkampfia*	Neg
FW08-60	None	*Hartmanella*	Neg
FW08-61	None	None	Neg
FW08-62	None	*Hartmanella, Vahlkampfia*	Neg
FW08-63	None	*Hartmanella*	Neg
FW08-64	None	None	Neg
FW08-65	None	None	Neg
FW08-66	None	None	Neg
FW08-67	None	*Acanthamoeba*	+
FW08-68	None	*Hartmanella*	+
FW08-69	None	*Hartmanella*	+
FW08-70	None	None	+
FW08-71	None	None	+

Continued:

Sample	Organisms cultured		Nested PCR
	N. fowleri	Others	*N. fowleri*
FW08-72	None	None	+
FW08-73	None	*Vahlkampfia*	+
FW08-74	None	None	+
FW08-75	None	None	+
FW08-76	None	*Vanella*	+
FW08-77	None	None	Neg
FW08-79	None	None	Neg
FW08-80	None	None	Neg
FW08-81	None	*Acanthamoeba*	Neg
FW08-82	None	Unknown	+
FW08-83	None	None	Neg
FW08-84	None	None	Neg
FW08-85	None	None	Neg
FW08-86	None	None	Neg
FW08-87	None	*Vanella*	Neg
FW08-88	None	Unknown	Neg
FW08-89	None	*Cashia*	Neg
FW08-90	None	*Cashia*	Neg
FW09-01	None	None	Neg
FW09-02	None	*Acanthamoeba*, Unknown	Neg
FW09-03	None	None	Neg
FW09-04	None	*Vanella*, Unknown	Neg
FW09-05	None	*Cashia*	Neg
FW09-06	None	*Acanthamoeba*	Neg
FW09-07	None	None	+
FW09-08	None	*Platyamoeba*	+
FW09-09	None	None	Neg

Continued:

Sample	Organisms cultured		Nested PCR
	N. fowleri	Others	*N. fowleri*
FW09-10	None	None	Neg
FW09-11	None	None	+
FW09-12	None	None	Neg
FW09-13	None	None	Neg
FW09-14	None	None	Neg
FW09-15	None	*Hartmanella*	Neg
FW09-16	None	*Hartmanella*, *Acanthamoeba*	Neg
FW09-17	None	None	Neg
FW09-18	None	*Platyamoeba*	Neg
FW09-19	None	*Acanthamoeba*	Neg
FW09-20	None	*Acanthamoeba*	Neg
FW09-21	None	None	Neg
FW09-22	None	None	Neg
FW09-23	None	*Acanthamoeba*, *Platyamoebae*	Neg
FW09-24	None	*Vanella*	Neg
FW09-25	None	None	Neg
FW09-26	None	None	Neg
FW09-27	None	None	Neg
FW09-28	None	*Acanthamoeba*, *Platyamoeba*	Neg
FW09-29	None	*Vanella*	Neg
FW09-30	None	None	Neg
FW09-31	None	*Vanella*	Neg
FW09-32	None	None	Neg
FW09-33	None	*Hartmanella*	Neg
FW09-34	None	None	+
FW09-35	None	*Hartmanella*	Neg

Continued:

Sample	Organisms cultured		Nested PCR
	N. fowleri	**Others**	***N. fowleri***
FW09-36	None	*Hartmanella*	Neg
FW09-37	None	*Hartmanella, Vanella*	Neg
FW09-38	None	None	Neg

clear water

water with sediment

water with algae

water with stones

5.2.4.2 Sensitivity and specificity of the nested *N. fowleri* primers

The sensitivity limit of the one-step nested PCR when tested with genomic *N. fowleri* DNA is shown in Figure 5.53. The lowest amount of genomic *N. fowleri* DNA that could be amplified with the one-step nested PCR was 10 pg (Lane 4), with only a single band at 506 bp observed on agarose gels. The specificity of the *N. fowleri* one-step nested PCR with DNA from other amoebae and *E. coli* are shown in Figure 5.54 and Figure 5.55. No bands were observed when the nested PCR was performed using DNA of *E. coli* (JM101), *A. castellanii* (ATCC 50370), *A. polyphaga* (CCAP 1501/3G), *A. polyphaga* (ATCC 30461), *A. polyphaga* (Ros), *A. hatchetti* (CDC V573), *A. castellanii* (ATCC 30234), *Acanthamoeba* spp. (SCSK09-01 to SCSK09-07), *N. gruberi* (ATCC 30224) and *B. mandrillaris* (ATCC 50209). Positive controls with *N. fowleri* plasmid DNA yielded 2 bands of 756 bp and 506 bp (Figure 5.54**,** Lane 6 and Figure 5.55, Lane 14).

Further specificity testing was performed using genomic DNA of an in-house *N. lovaniensis* (strain C0490). The identity of the C0490 strain was confirmed by PCR amplification using primers (NL5F/R and NF2F/R, See Table 2.3) of *N. lovaniensis*-*N.*

fowleri duplex PCR. No band at 300 bp was observed when the DNA was amplified using the *N. fowleri* specific primers (NF2F/R) and combination of both set of primers (NL5F/R-NF2F/R) (Figure 5.56, Lanes 2 and 6). Amplification of *N. lovaniensis* DNA (strain C0490) using *N. lovaninesis* specific primers (NL5F/R) and combination of both set of primers (NL5F/R-NF2F/R) yielded a single band of 650 bp (Figure 5.56, Lanes 4 and 6). *N. fowleri* one-step nested PCR using *N. fowleri* plasmid DNA (Figure 5.56, Lane 9) showed two bands of the expected size (765 bp and 506 bp), while no DNA amplification was seen with the *N. lovaniensis* DNA (Figure 5.56, Lane 8).

The *N. fowleri* one-step nested PCR was also performed using genomic DNA extracted from trophozoites of four *N. fowleri* strains (CCAP 1518, KUL ATCC 30808, NH1 and MSM). All strains gave a band at 506 bp from PCR amplification using the nested primers (Figure 5.57, Lanes 2 to 5). Only two *N. fowleri* strains (CCAP 1518 and KUL ATCC 30808), showed a band correspond to the external primers with the expected size of 767 bp (Figure 5.57, Lanes 2 and 3).

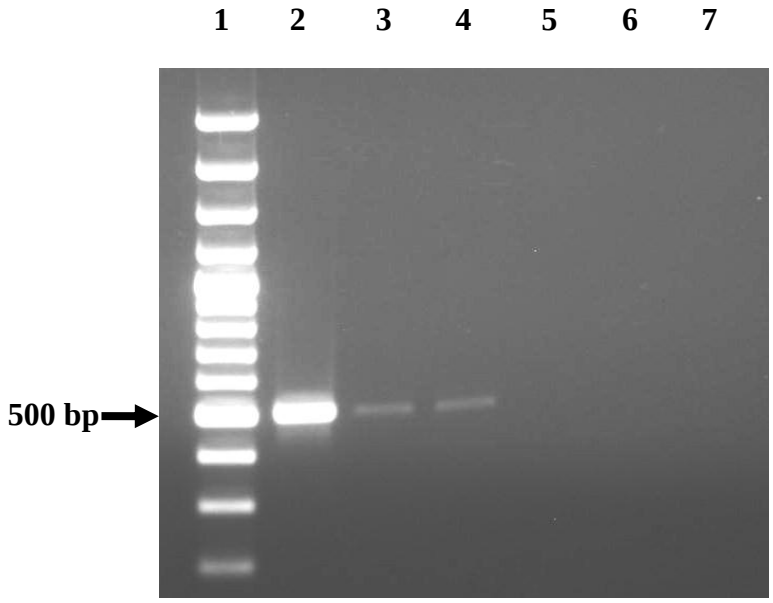


Figure 5.53 Sensitivity of *N. fowleri* one-step nested PCR with various amounts of genomic *N. fowleri* DNA (ATCC 30808). Lane 1: 100 bp marker; Lane 2: 1ng; Lane 3: 100 pg; Lane 4: 10 pg; Lane 5: 1 pg; Lane 6: 100 fg; Lane 7: Negative control (nanopure water)

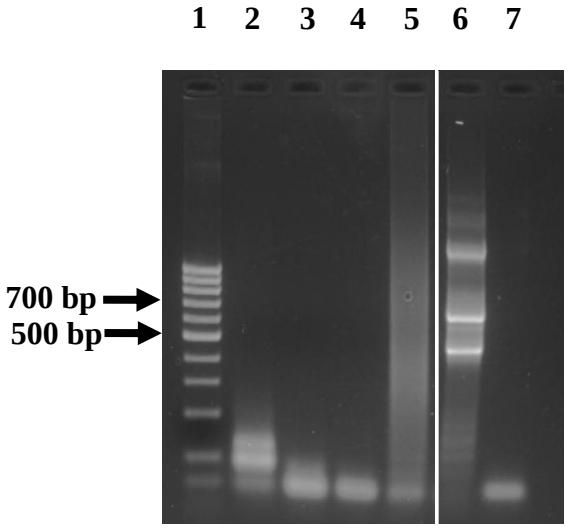


Figure 5.54 Specificity of the one-step nested *N. fowleri* PCR. Lane 1: 100 bp marker; Lane 2: *E.coli* strain JM101; Lane 3: *A. castellanii* strain (ATCC 50370); Lane 4: *A. polyphaga* strain CCAP 1501/3G; Lane 5: *B. mandrillaris* (ATCC 50209); Lane 6: (Positive control) *N. fowleri* plasmid DNA; Lane 8: Negative control

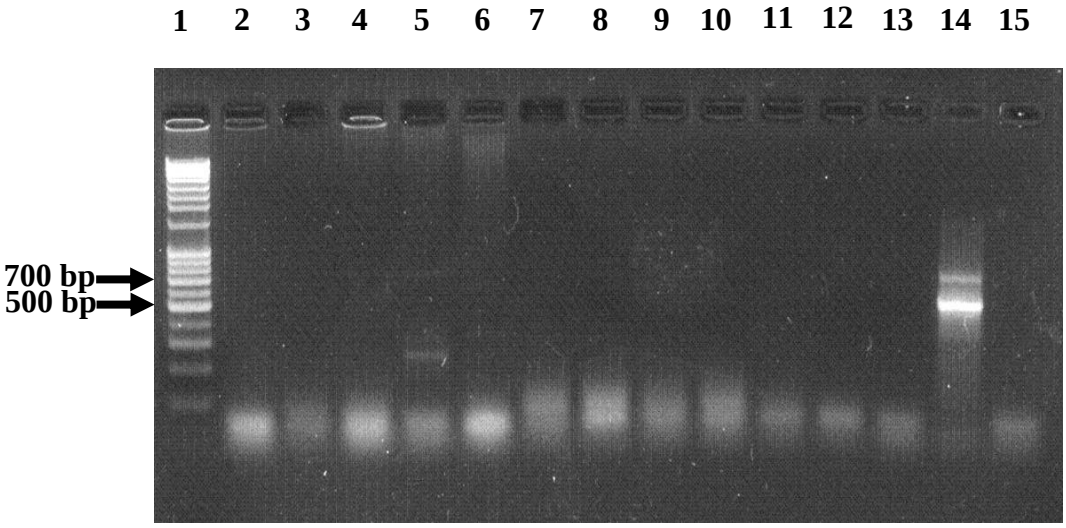


Figure 5.55 Specificity of one-step nested *N. fowleri* PCR against other amoebae. Lane 1: 100 bp marker; Lane 2: *N. gruberi* (ATCC 30224); Lane 3: *A. polyphaga* (ATCC 30461); Lane 4: *A. polyphaga* (Ros); Lane 5: *A. hatchetti* (CDC V573); Lane 6: *A. castellanii* (ATCC 30234); Lane 7: *Acanthamoeba* sp. (SCSK09-01); Lane 8: *Acanthamoeba* sp. (SCSK09-02); Lane 9: *Acanthamoeba* sp. (SCSK09-03); Lane 10: *Acanthamoeba* sp. (SCSK09-04); Lane 11: *Acanthamoeba* sp. (SCSK09-05); Lane 12: *Acanthamoeba* sp. (SCSK09-06); Lane 13: *Acanthamoeba* sp. (SCSK09-07); Lane 14: Positive control (*N. fowleri* plasmid DNA 1:200); Lane 15: Negative control (nanopure water)

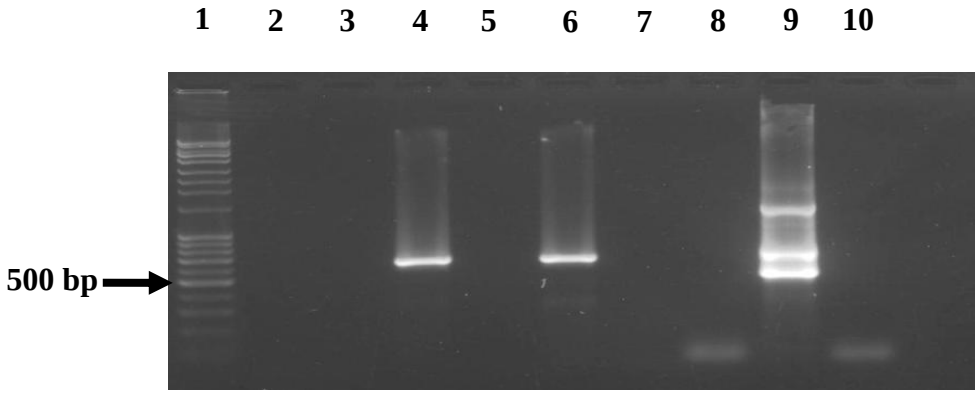


Figure 5.56 Amplification of *N. lovaniensis* genomic DNA (strain C0490) using primers of *N. lovaniensis*-*N. fowleri* duplex PCR and *N. fowleri* one-step nested PCR. Lane 1: 100 bp marker; Lane 2: *N. lovaniensis* + NF2F/R primers (specific for *N. fowleri*); Lane 3: Negative control (nanopure water); Lane 4: *N. lovaniensis* + NL5F/R primers (specific for *N. lovaniensis*); Lane 5: Negative control (nanopure water); Lane 6: *N. lovaniensis* + NL2F/R and NL5F/R primers; Lane 7: Negative control (nanopure water); Lane 8: *N. lovaniensis* + one-step nested primers; Lane 9: *N. fowleri* + one-step nested primers; Lane 10: Negative control (nanopure water)

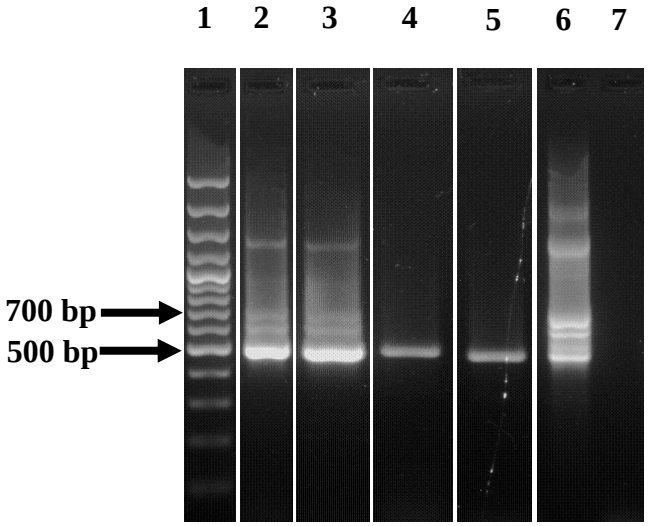


Figure 5.57 *N. fowleri* one-step nested PCR using genomic DNA extracted from trophozoites of *N. fowleri*. Lane 1: 100 bp marker; Lane 2: *N. fowleri* DNA (CCAP 1518); Lane 3: *N. fowleri* DNA (KUL ATCC 30808); Lane 4: *N. fowleri* DNA (NH1); Lane 5: *N. fowleri* DNA (MSM); Lane 6: *N. fowleri* plasmid DNA; Lane 7: Negative control (nanopure water)

5.2.5 Detection of *Acanthamoeba* DNA from environmental samples by the developed DNA extraction method and PCR

As most of the cultivation results were positive for *Acanthamoeba,* a total of 67 soil samples were tested for PCR amplification using *Acanthamoeba* 18S rDNA primers (Schroeder *et al.*, 2001). This experiment was also to investigate the efficacy of the DNA extraction method in extracting DNA from eukaryotic cells. A total of 39 (58%) samples were positive by PCR and the summary of the results is shown in Table 5.9. Soil samples from Italy showed the highest percentage (67%) of samples positive for *Acanthamoeba* DNA. This was followed by soils from Southern California (65%), United Kingdom (62%), South Africa (50%) and Portugal (25%). As shown in Figure 5.58, the positives samples from Italy were IT09-01 (Lane 2), IT09-03 (Lane 4), IT09-04 (Lane 5) and IT09-05 (Lane 6). The 11 positive samples from Southern California (Figure 5.59) were SC09-02 (Lane 3), SC09-03 (Lane 4), SC09-05 (Lane 6), SC09-07 (Lane 8), SC09-08 (Lane 9), SC09-09 (Lane 10), SC09-10 (Lane 11), SC09-11 (Lane 12), SC09-12 (Lane 13), SC10-04 (Lane 20) and SC10-05 (Lane 21). From the total of 21 positive soils samples from the United Kingdom, 13 were from the East Midlands (EM08-01, EM08-02, EM08-03, EM08-04, EM08-05, EM08-06, EM08-07, EM08-09, EM08-12, EM08-13, EM08-14, EM08-15 and EM08-16) and 8 from the University of Reading farm (UR09-01, UR09-02, UR09-03, UR09-04, UR09-10, UR09-11, UR09-12 and UR09-13). Representatives of the PCR product are shown in Figure 5.60 to Figure 5.62. For South African soils, the one which was positive for *Acanthamoeba* DNA was collected from Kruger Park (SA08-02). Only 2 (PT09-08 and PT09-09) out 8 samples collected in Portugal were positive for *Acanthamoeba* as shown in Figure 5.63.

Table 5.9 Detection of *Acanthamoeba* DNA from environmental samples by PCR

Country	**No. of sample tested**	**No. of samples positive by *Acanthamoeba* 18S rDNA PCR**	**Percentage of positive samples (%)**
United Kingdom	34	21	62
South Africa	2	1	50
Portugal	8	2	25
Italy	6	4	67
Southern California	17	11	65

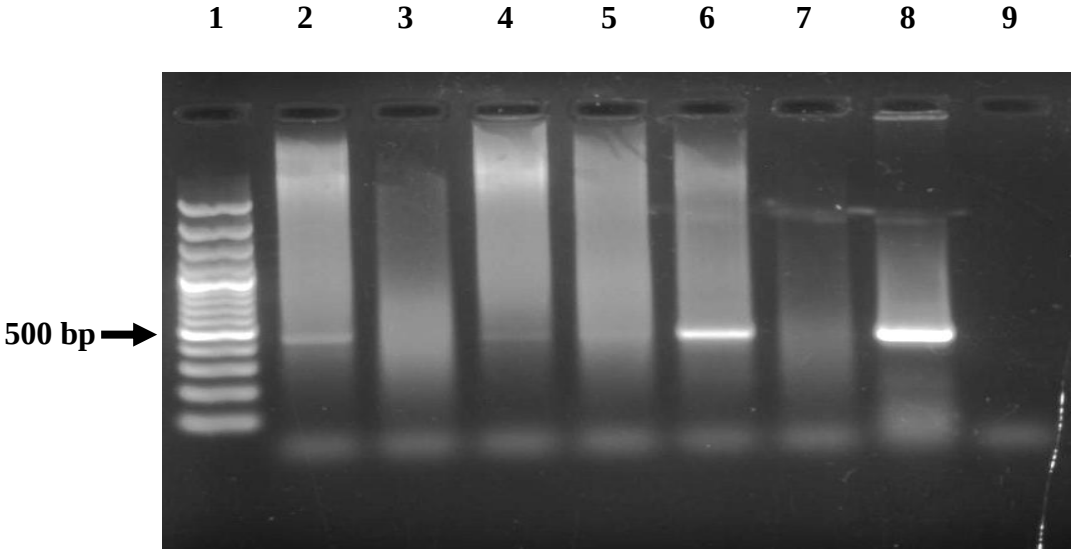


Figure 5.58 PCR amplification using *Acanthamoeba* 18S rDNA primers with Italian soils. Lane 1: 100 bp marker; Lane 2: IT09-01; Lane 3: IT09-02; Lane 4: IT09-03; Lane 5: IT09-04; Lane 6: IT09-05; Lane 7: IT09-06; Lane 8: Positive control (*Acanthamoeba* DNA); Lane 9: Negative control (nanopure water)

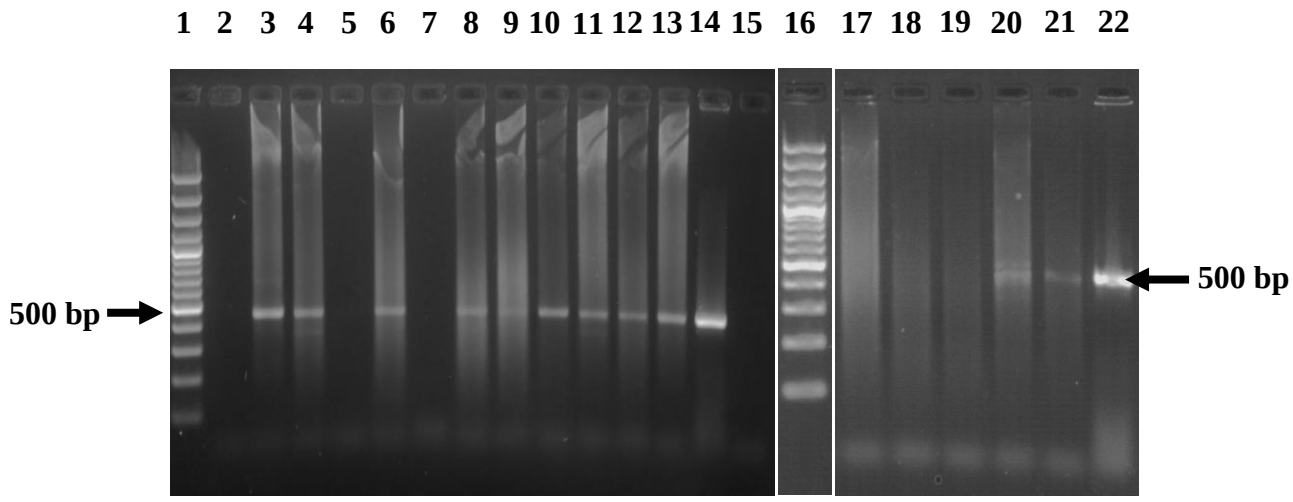


Figure 5.59 PCR amplification using *Acanthamoeba* 18S rDNA primers with Southern Californian soils. Lanes 1 and 16: 100 bp marker; Lane 2: SC09-01; Lane 3: SC09-02; Lane 4: SC09-03; Lane 5: SC09-04; Lane 6: SC09-05; Lane 7: SC09-06; Lane 8: SC09-07; Lane 9: SC09-08; Lane 10: SC09-09; Lane 11: SC09-10; Lane 12: SC09-11; Lane 13: SC09-12; Lanes 14 and 22: Positive control (*Acanthamoeba* DNA); Lane 15: Negative control (nanopure water); Lane 17: SC10-01; Lane 18: SC10-02; Lane 19: SC10-03; Lane 20: SC10-04; Lane 21: SC10-05

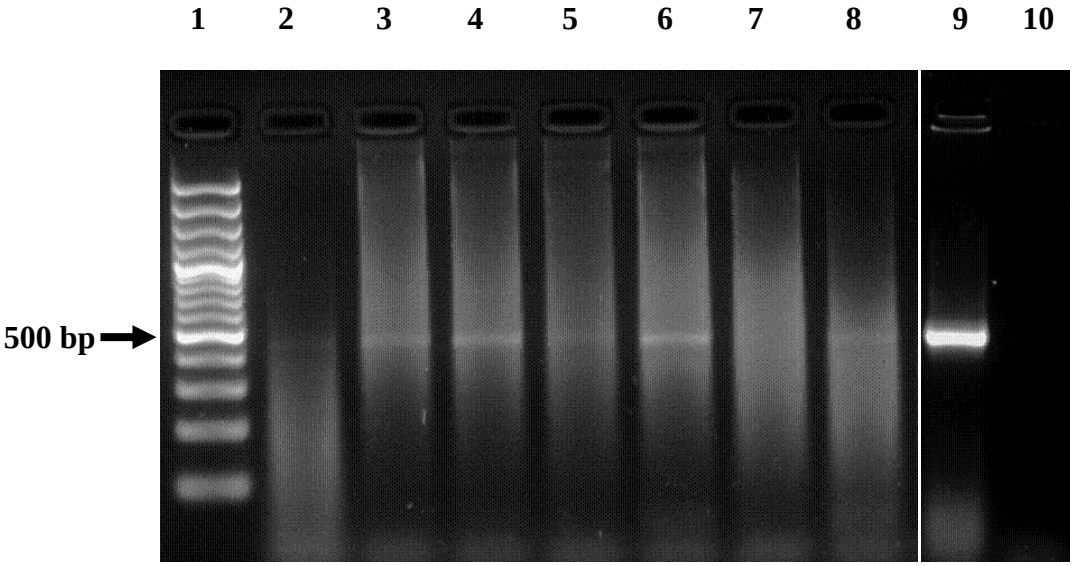


Figure 5.60 PCR amplification using *Acanthamoeba* 18S rDNA primers with East Midlands, UK soils. Lane 1: 100 bp marker; Lane 2: EM08-03; Lane 3: EM08-04; Lane 4: EM08-05; Lane 5: EM08-06; Lane 6: EM08-07; Lane 7: EM08-08; Lane 8: EM08-09; Lane 9: Positive control (*Acanthamoeba* DNA); Lane 10: Negative control (nanopure water)

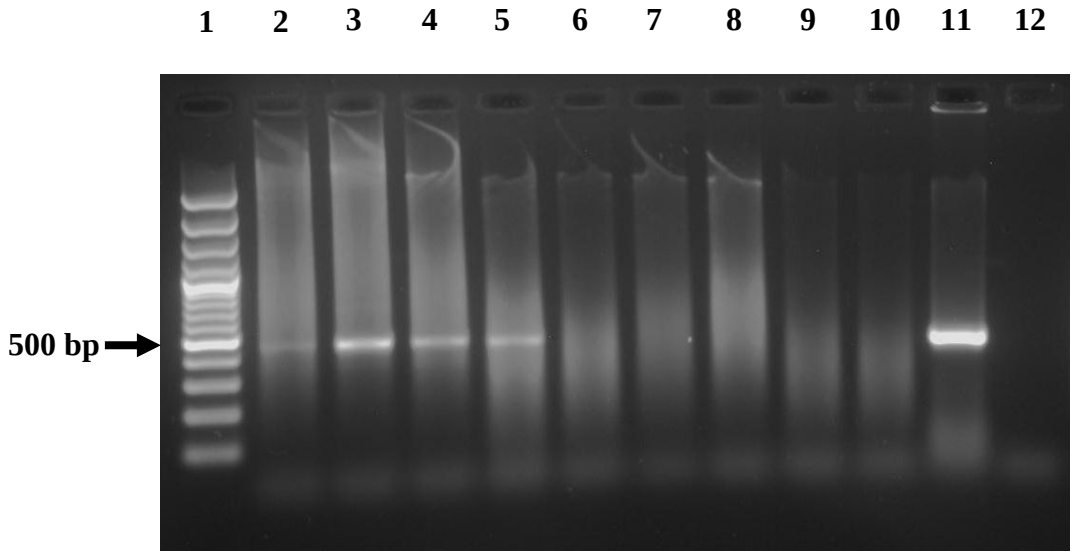


Figure 5.61 PCR amplification using *Acanthamoeba* 18S rDNA primers with University of Reading, UK soils (0-10 cm). Lane 1: 100 bp marker; Lane 2: UR09-01; Lane 3: UR09-02; Lane 4: UR09-03; Lane 5: UR09-04; Lane 6: UR09-05; Lane 7: UR09-06; Lane 8: UR09-07; Lane 9: UR09-08; Lane 10: UR09-09; Lane 11: Positive control (*Acanthamoeba* DNA); Lane 12: Negative control (nanopure water)

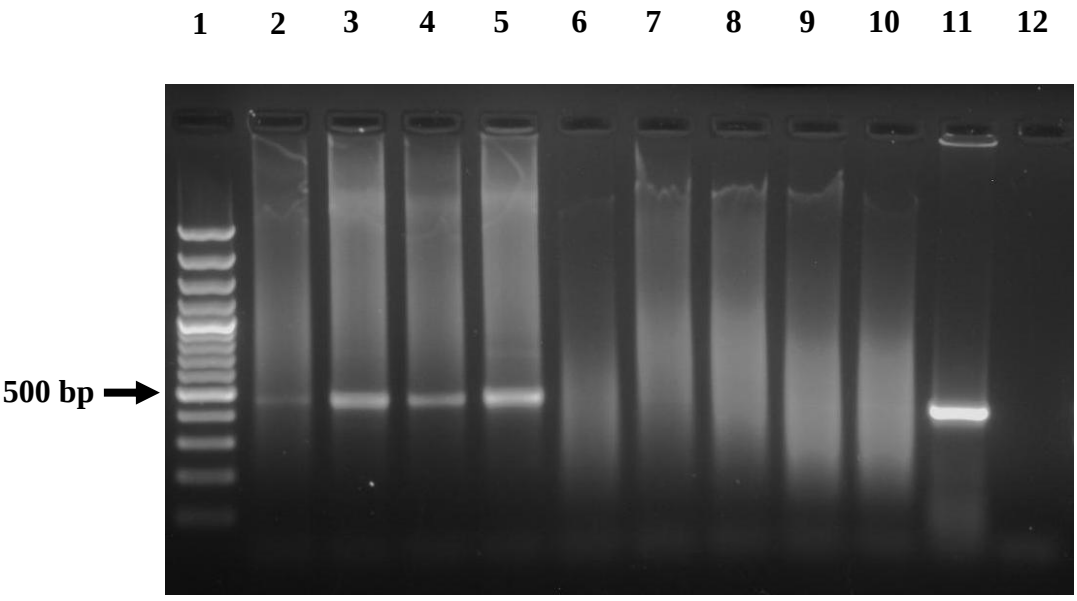


Figure 5.62 PCR amplification using *Acanthamoeba* 18S rDNA primers with University of Reading, UK soils (10-20 cm). Lane 1: 100 bp marker; Lane 2: UR09-10; Lane 3: UR09-11; Lane 4: UR09-12; Lane 5: UR09-12; Lane 6: UR09-14; Lane 7: UR09-15; Lane 8: UR09-16; Lane 9: UR09-17; Lane 10: UR09-18; Lane 11: Positive control (*Acanthamoeba* DNA); Lane 12: Negative control (nanopure water)

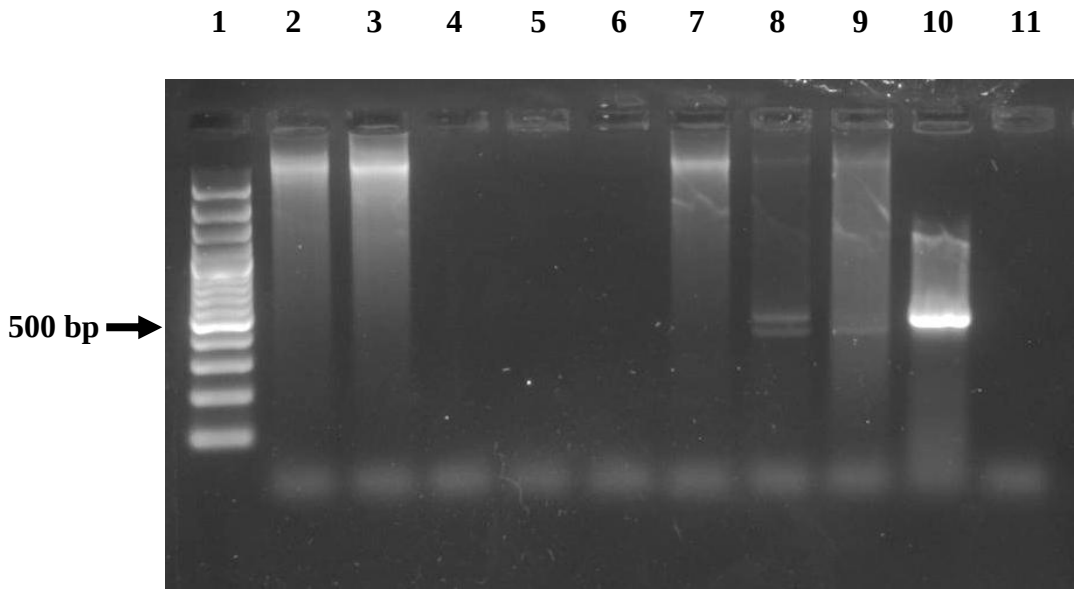


Figure 5.63 PCR amplification using *Acanthamoeba* 18S rDNA primers with Portugal soils. Lane 1: 100 bp marker; Lane 2: PT09-01; Lane 3: PT09-02; Lane 4: PT09-03; Lane 5: PT09-04; Lane 6: PT09-05; Lane 7: PT09-06; Lane 8: PT09-07; Lane 9: PT09-08; Lane 10: Positive control (*Acanthamoeba* DNA); Lane 11: Negative control (nanopure water)

5.3 Discussion

5.3.1 Detection of *B. mandrillaris* from environmental samples using the developed extraction method and a nested PCR

Studies on the ecological and geographical distribution of *B. mandrillaris* have been hampered by the inability of the amoeba to feed on bacteria. This has made the environmental isolation of *B. mandrillaris* using the conventional NNA-*E. coli* plates impossible or laborious process due to overgrowth by other amoebae and contaminating fungi which means constant monitoring and regular subculturing (Rideout *et al.*, 1997, Schuster *et al.*, 2003). In this chapter, it was shown that the ecological distribution of *B. mandrillaris* could be studied using a developed nested PCR and environmental DNA extracted directly from soil and water samples, without the need of primary culture.

A total of 186 environmental samples have been extracted using the developed DNA extraction method. The purified DNAs were confirmed to have PCR inhibitors levels that were not sufficiently high to inhibit the PCR reactions as shown by the positive amplification of prokaryotic 16S rDNA for all of the environmental samples tested, although 19 samples were only positive upon reamplification of the primary PCR product. It is speculated that the inability of the 19 purified DNAs to be amplified during the first round of PCR might not be due to the humic substances but possibly low concentration of target DNA. This is supported by a study which showed that unpurified DNA containing humic substances would not give any visible product even after two sequential PCR amplifications (Young *et al.*, 1993). In addition, Tsai and Olson have shown that a tiny volume (1 µl) of undiluted humic-acid-like extract can interfere with PCR amplification, no matter what amount of DNA was present (Tsai *et al.*, 1992).

The advantage of using 18S rRNA gene as a target for amplification is that it is present in multiple copies in each cell (Byers *et al.,* 1990, Clark *et al.,* 1987). The multiple copies will provide more suitable target DNA for amplification, making the PCR assays more sensitive (Qvarnstrom *et al.*, 2006). The gene has also been reported to be evolutionarily stable with limited intra-species nucleotides variation (Criado *et al.*, 2006). Hence it is suitable to be used for detection of *B. mandrillaris*, which at present is the only species within the genus (Visvesvara *et al.*, 2007). In this study, it was shown that the use of nested PCR has greatly improved the numbers of positive samples detected as compared to the number obtained with the primary PCR using only the external primers. This could be due to the improved sensitivity limit of the nested PCR, as shown by the positive amplification using 10 spiked trophozoites or 0.1 pg of genomic DNA, in contrast to those shown by the primary PCR with sensitivity limit of 100 spiked trophozites and 1 pg of genomic DNA.

It was revealed in the study reported here that *B. mandrillaris* DNA was detected in soils in Southern Californian and South Africa in contrast to none from the United Kingdom environmental samples. This suggested the amoeba has preference for warmer regions. In addition, the fact that all of the positive soil samples were collected from housing areas or road sides shows that their presence in the environments is not dependent on any special type of soil. *Balamuthia* amoebic encephalitis cases have been reported globally but almost 60% were from the United States with majority of the cases were reported in the Southern California (CDC, 2008a, Schuster *et al.*, 2009). Out of these, 25% were identified in California with 10 cases reported by the California Encephalitis Project (CEP) and five by the Centres for Disease Control (CDC). It was shown that 70% of the BAE cases detected by the CEP were from Southern California (CDC, 2008a, Schuster *et al.*, 2009). The average temperature in Southern California is 17°C and in summer can reach up to 29°C (Fairmont, 2011). This finding was in accordance with the high number of positive

samples detected from the Southern Californian samples (94%), as shown by the developed nested PCR used in this study.

In the United States, BAE cases in humans have commonly been associated with Hispanic ethnicity (Schuster *et al.*, 2004a, Schuster *et al.*, 2009). Based on the United States Census Bureau statistics for 2000, 12.5% of the US population are Hispanics while in the state of California 32% are Hispanics. However, the BAE cases related to Hispanics was about 50% in the US and more than 70% in California (Schuster *et al.*, 2004a). The high incidence of BAE in the Hispanics could be due to their frequent involvement in agriculture work which may expose them to infection through injuries or inhalation of dust containing the amoeba (Schuster *et al.*, 2004a). It has been reported in the 2002 Census of agriculture that 50,592 farms in the US had people of Hispanic origin as the main workers. In the California alone, the total Hispanic farms workers were 11,985 (USDA *et al.*, 2004). In addition to being due to the agriculture work, the high numbers of BAE in the Hispanics may possibly due to genetic predisposition (Schuster *et al.*, 2004a). Previously, in a survey of *Acanthamoeba* antibodies in healthy humans, only 40% of Hispanics serum samples showed reactivity against *A. polyphaga* compared to ≥ 90% positive serum samples by each of the other three ethnic groups studied (Caucasians, Asian and AfricanAmericans) (Chappell *et al.*, 2001). Hence, the Hispanics may as well produce low levels of antibodies against *B. mandrillaris*, which make them more susceptible to infections.

The result which showed that only one out of eight Portugal soil samples was positive for *B. mandrillaris* was in accordance with the limited BAE cases reported in the country. To date, the only reported BAE case in Portugal that had a fatal consequence was in a young male (8 years old) (Tavares *et al.*, 2006). The source of infection was speculated to be due to swimming in pools or outdoor plant collecting activity (Tavares *et al.*, 2006). However, as Portugal is a warm country with an average temperature of 17°C (climatemp.info,

2011), there might be other undefined factors that may contribute to their geographical distribution besides the high temperature. The detection of *B. mandrillaris* in 100% of the South African soils confirms the preference of the amoeba to regions with high temperatures. Currently, no reports on the presence of *B. mandrillaris* or BAE cases in South Africa are available.

In experiments to determine the presence of *B. mandrillaris* in aquatic environments, the detection of the amoeba in 53% of French river samples showed that thermally polluted water due to effluent from a nearby power station might also be a preferred habitat. This finding might explain the BAE infection in two dogs with histories of swimming in stagnant ponds in Australia and California (Foreman *et al.*, 2004, Finnin *et al.*, 2007).

5.3.2 Detection of *N. fowleri* from water samples using the developed DNA extraction method and a one-step nested PCR

Nested PCR has recently been developed and employed for the detection of *N. fowleri* from both natural and thermally enriched aquatic environments (Reveiller *et al.*, 2002, Maclean *et al.*, 2004). A typical nested PCR involves primary DNA amplification using outer primers and the PCR products obtained act as templates for the inner primers in a secondary PCR. As a consequence, this can significantly improve the detection sensitivity and specificity of the assay and mitigate the effect of Taq polymerase inhibitors that are present in the extracted DNA. Generally, a nested PCR is performed in two separate reactions, with a sample of the primary product of the first round of reaction is being transferred into a separate second tube. This may lead to increase in the occurrence of false positive reactions due to cross-contamination during the transferring step. The advantage of the one-step nested PCR used in this present study is it enabled the reaction to be conducted in a single tube, which greatly simplifies the procedure and reduces the risk of cross-

contamination. The difference in the annealing temperatures (Tm) of the outer and inner primer sets allowed each primer set to react independently in the first and second round of the assay. In this study, the detection of *N. fowleri* from water samples was performed using environmental DNA extracted using a reliable method as described in Chapter 4 and in combination with the one-step nested PCR.

In the UK, any manipulation of live *N. fowleri* has to be done in a Category 3 containment lab and is not allowed in the normal laboratory. However, to obtain involves many stages, such as requirement of medical check-up, preparation of defined standard operating procedures, approval from the board and training lasting between 6 months to 1 year. It was concluded, therefore, that it was not practical to do this within the available time. Therefore, the detection of *N. fowleri* with the one-step nested PCR in this study was performed using *N. fowleri* plasmid DNA as the positive control rather than the genomic DNA from live amoeba. Although the nested PCR assay has been claimed to be specific only for *N. fowleri* (personal communication from Dr Simon Kilvington), towards the end of the experiment it was decided to confirm the specificity of the assay. In determining the ability of the nested PCR to differentiate *N. fowleri* from another thermophilic *Naegleria,* two *N. lovaniensis* strains (ATCC 30569 and an in-house strain C0490) were used. However, the ATCC strain failed to grow in the laboratoty and therefore only strain C0490 was tested. The identity of the strain C0490 was initially confirmed as *N. lovaniensis* by PCR using primers of *N. lovaniensis-N. fowleri* duplex PCR that was performed separately using primer set of NL5F/R (*N. lovaniensis*) and NF2F/R (*N. fowleri*) as individual pairs and a combination of both set of primers (NL5F/R and NF2F/R). PCRs using the NL5F/R and NL5F/R-NF2F/R showed products with the expected size of 650 bp, confirmed that the strain C0490 DNA was of *N. lovaniensis*. In contrast, no band was observed at 300 bp (expected size for *N. fowleri*) using the NF2F/R and NL5F/R-NF2F/R primers of the *N.*

lovaniensis-N. fowleri duplex PCR. No amplification was seen when the genomic DNA of *N. lovaniensis* strain C0490 was tested with the one-step nested PCR developed in this study. Genomic DNA from four strains of *N. fowleri* was eventually tested with the one-step nested PCR. Positive amplification with PCR product of 506 bp shown by all the *N. fowleri* strains confirmed that the one-step PCR is specific for the detection of *N. fowleri*.

Previously, Reveiller *et al*. (2002) reported a *N. fowleri* nested PCR assay as having a sensitivity limit of 5 pg of DNA (approximately 25 amoebae) or 5 whole amoebae when spiked into PCR-grade water (Reveiller *et al.*, 2002). The one-step nested PCR of the study in this thesis, had a positive reaction with 10 pg of purified *N. fowleri* DNA but not with 1 pg. Because the sensitivity limit of the PCR assay lies between 1 and 10 pg of DNA, it is probably comparable to that reported previously by Reveiller *et al*. (2002). Spiking of samples with whole amoebae was not done in this study because it was believed that the sensitivity of the PCR may have been decreased by inhibitory substances that may co-purify during the extractions. The study by Reveiller *et al*. (2002) showed positive PCR amplification when 75 *N. fowleri* trophozoites were spiked in tap, river or lake water samples but they did not tested with lower numbers of amoebae. The possibility that the positive results with the water samples may have resulted from amplification of DNA from dead *N. fowleri* cannot be excluded. Nevertheless, knowledge that an environment may contain *N. fowleri*, even in the non-culturable state, would still be of importance for ecological and public health studies.

The reported sensitivity of nested PCR assays for detection of *N. fowleri* in environmental samples has been shown with and without initial culture enrichment of the samples (Maclean *et al.*, 2004, Marciano-Cabral *et al.*, 2003b, Pelandakis *et al.*, 2002). In this study, a combination of the one-step *N. fowleri* nested PCR and the developed

environmental DNA extraction method showed better sensitivity than culture. *N. fowleri* was not detected from water samples collected in France and the UK on culture analysis but 18/119 (15%) were shown to be positive by the one-step nested PCR, all from the French waters. *N. fowleri* has been detected previously from both sites by culture and the reasons that negative results that were obtained in the present study are uncertain (Kilvington *et al.*, 1995b, Kilvington *et al.*, 1997, Pelandakis *et al.*, 2002). It has been reported that culture of French samples on NNA-*E. coli* plates in the preceding year also were negative for *N. fowleri* and the UK site has not been investigated since 1997 (Kilvington *et al.*, 1997). This suggests that the presence of *N. fowleri* has decreased in the waters or that it varies irregularly in small numbers (Kilvington *et al.*, 1995b, Kilvington *et al.*, 1997, Pelandakis *et al.*, 2002). Another explanation may be the amount of material that can be analysed by the two methods. Thus only small amounts of material (< 1 gram) can be cultured on NNA-*E. coli* plates whereas up to 10 grams can be analysed using the direct DNA extraction developed in this study. Furthermore, the presence of *N. fowleri* can be hidden by the occurrence of faster growing FLA and dependence on the flagellation test to screen for *Naegleria* spp. may not be reliable, resulting in false negative results (Behets *et al.*, 2003, De Jonckheere *et al.*, 2001)

In this study, a reliable and sensitive one-step nested PCR method for detecting *N. fowleri* directly from environmental samples has been utilised and assessed. Upcoming developments in the technology may include evaluation of quicker DNA extraction from environmental samples and the use of real-time PCR methods that could also enumerate the occurrence of *N. fowleri* in the processed samples and distinguish viable from dead organisms (Qvarnstrom *et al.*, 2006, Robinson *et al.*, 2006). Even though it was revealed in this study that the nested PCR was more sensitive than culture for the detection of *N. fowleri*, culture isolation should always be attempted in conjuction, as the ability to

perform molecular typing assays on isolates is valuable in studying the genetic diversity of *N. fowleri* and in epidemiological analyses to recognise sources of infection (Kilvington *et al.*, 1995b, Pelandakis *et al.*, 2002, van Belkum *et al.*, 1992).

5.3.3 Detection of *Acanthamoeba* from soil samples using the developed DNA extraction method and PCR with published primers

Acanthamoeba is the most commonly found free-living amoeba and has been detected in soils, aquatic habitats and air (Rodriguez-Zaragoza, 1994, Visvesvara *et al.*, 2007). Due to the ability of the amoeba to feed on bacteria, the amoeba can easily be isolated from environmental samples using the conventional culture method with NNA plates that have been lawned with *E. coli* as the food source (Page, 1988). As described in Section 5.2.2, most of the soil samples (93%) were found to contain *Acanthamoeba*, using the conventional culture method and incubation at 32°C. In order to test the robustness of the developed DNA extraction method (Chapter 4) in detecting other eukaryotic organisms, DNA from soil samples were subjected for PCR using published primers that are specific for the *Acanthamoeba* genus (Schroeder *et al.*, 2001).

A total of 58% soil samples tested by PCR were positive for *Acanthamoeba*. The positive samples were of soils collected from five different countries, showing the worldwide distribution of *Acanthamoeba*. The difference in the numbers of positive samples between the culturing and PCR methods could be due to the culturing method which allows *Acanthamoeba* to proliferate following feeding on the *E. coli*, thus allowing sufficient numbers of the amoeba to be detected after a few days of culturing on the NNA-*E. coli* plates. In contrast, the PCR was performed using DNA that was extracted directly from the soil samples without primary culturing. Therefore, the numbers of *Acanthamoeba* present in the soil might not be sufficient for PCR. Nevertheless, the *Acanthamoeba* PCR with

direct DNA extraction method has the advantage over the culturing method in that faster positive results could be obtained and hence would be more suitable for use in ecological studies involving large numbers of samples. The culture method may then be used for confirmation of samples that are *Acanthamoeba*-negative by PCR.

6 Production and use of poly- and monoclonal antibodies against *Balamuthia mandrillaris*

6.1 Introduction

As discussed in Section 1.5, polyclonal antibodies have been developed against *Acanthamoeba* spp., *N. fowleri*, non-pathogenic *Naegleria* spp. and *B. mandrillaris*, and these have been used in immunofluorescence assays, particularly for the confirmation of the presence of these amoebae in clinical samples. However, polyclonal antibodies suffer from a lack of specificity due to cross-reactivity problems (Flores *et al.*, 1990, Guarner *et al.*, 2007). To overcome this problem, attention has turned to monoclonal antibodies.

Monoclonal antibodies against *N. fowleri* and *Acanthamoeba* spp. have been made and have been used in a variety of immunological assay methods (Sparagano *et al.*, 1993, Reveiller *et al.*, 2000, Turner *et al.*, 2005). As with polyclonal antibodies, monoclonal antibodies against *Acanthamoeba* and *N. fowleri* have been successfully used for confirmation of the presence of these amoebae in clinical materials (Visvesvara *et al.*, 1987, Flores *et al.*, 1990, Reveiller *et al.*, 2003, Turner *et al.*, 2005). Until now, no monoclonal antibodies against *B. mandrillaris* have been reported. This chapter reports the results of work in which both poly- and monoclonal antibodies to *B. mandrillaris* were to be produced and their specificity against the amoeba determined. In addition, the use of the antibodies in immunomagnetic assay was investigated for their suitability in facilitating the separation of the amoeba from environmental samples.

6.2 Results

6.2.1 Production of *B. mandrillaris* polyclonal antibodies in mice

Three mice (PSBM-1, PSBM-2, and PSBM-3) were initially immunised with sonicated *B. mandrillaris* trophozoites to determine whether the prepared immunogen could be used to develop anti-*B. mandrillaris* antibodies in the experimental animals. The presence of anti-*B. mandrillaris* antibodies was checked by ELISA using serum of mouse PSBM-3, obtained 2 weeks after the final immunisation, and compared with pre-immune serum of the same mouse obtained before the immunisation schedule. The sera from mice PSBM-1 and PSBM-2 were not tested by ELISA but were kept at -80°C for further use. A positive antigen-antibody reaction by ELISA was indicated by a blue coloration, while a negative antigen-antibody reaction was seen as colourless. Sulphuric acid (0.18M, H_2SO_4) was used to stop further antigen-antibody reaction and the addition of this solution caused changes of the blue colouration to yellow. As shown in Figure 6.1, using either the blue indicator colour (Row A) or yellow indicator colour (Row C), the post-immune serum of mouse PSBM-3 was positive up to a dilution of 1:6400 (Well 8). With both reactions, the colour started to fade at a dilution of 1:12,800 (Well 9) and very light colouration was seen at a dilution of 25,600 (Well 10). No antigen-antibody reaction was seen when dilutions of pre-immune serum of mouse PSBM-3 were reacted with the sonicated *B. mandrillaris* (Rows B and D).

The presence of anti-*B. mandrillaris* antibodies was also checked using Western blotting, performed using a single dilution (1:1000) of post- and pre-immune sera of mouse PSBM-3 with different concentrations of *B. mandrillaris* antigen. The results using post-immune serum are shown in Figure 6.2A. Strong bands at approximately 32 kD, 45 kD, 50 kD, 60 kD, 75 kD, 100 kD and 250 kD were seen against *B. mandrillaris* antigen concentration of

4 mg/ml (Lane 1a). When 0.4 mg/ml of *B. mandrillaris* antigen was used, 2 clearly visible bands at 50 kD and 60 kD but other faintly staining bands of other sizes could be seen in the track (Figure 6.2A, Lane 1b). No visible bands were observed for *B. mandrillaris* Ag concentrations of 0.04 mg/ml (Lane 1c) and 0.004 mg/ml (Lane 1d). Pre-immune serum of mouse PSBM-3 showed negative results against all concentrations of *B. mandrillaris* antigen tested (Figure 6.2B, Lanes 1a to 1d).

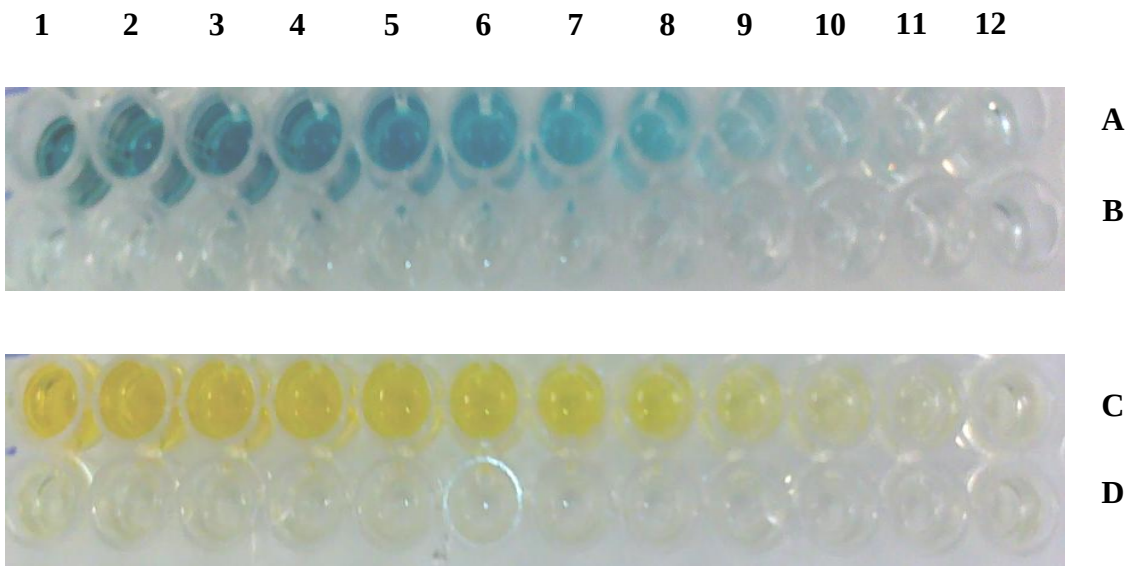


Figure 6.1 ELISA results for post- and pre-immune sera of mouse PSBM-3 against sonicated *B. mandrillaris*. Rows A and B: Colour reaction for post-immune (A) and pre-immune (B) sera before the addition of sulphuric acid; Rows C and D: Colour reaction for post-immune (C) and pre-immune (D) sera after the addition of sulphuric acid. Well 1: 1/50; Well 2: 1/100; Well 3: 1:200; Well 4: 1:400; Well 5: 1/800; Well 6: 1/1600; Well 7: 1/3200; Well 8: 1/6400; Well 9: 1/12,800; Well 10: 1/25,600; Well 11: 1/51,200; Well 12: 1/102,400

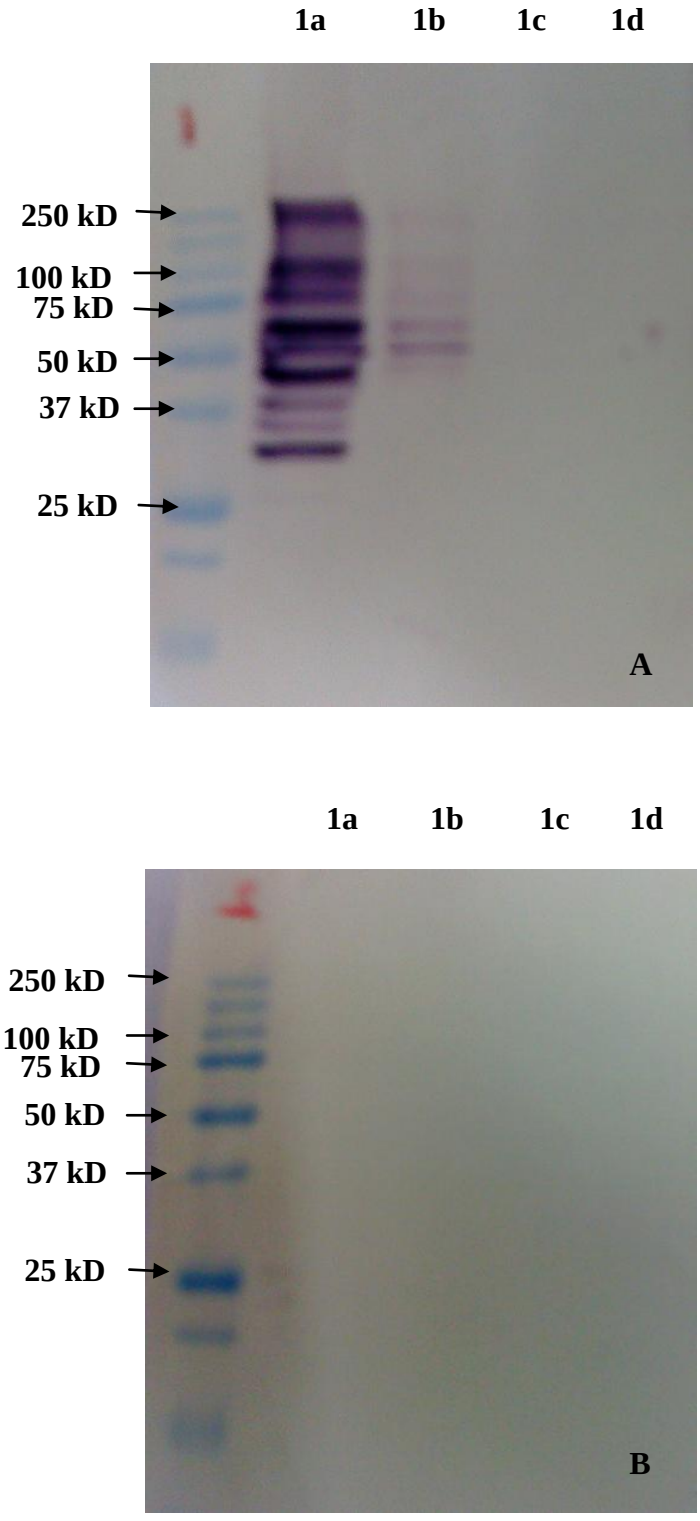


Figure 6.2 Western blot of post (A) and pre-immune (B) sera from mouse PSBM-3 against different concentrations of sonicated *B. mandrillaris* trophozoites. 1a: 4 mg/ml; 1b: 0.4 mg/ml; 1c: 0.04 mg/ml; 1d: 0.004 mg/ml. Each well was loaded with 20 µl of the respective concentration of protein.

6.2.2 Production of large-scale *B. mandrillaris* polyclonal antibodies in rabbits

Production of large-scale *B. mandrillaris* polyclonal antibodies was performed in two rabbits (UK4237 and UK4238) at Cambridge Research Biochemicals, using formalin-killed *B. mandrillaris* trophozoites. Based on discussions with the company, use of live microorganisms was not allowed and it was suggested that formalin-killed *B. mandrillaris* be used as it would be more suitable for subsequent detection purposes than sonicated *B. mandrillaris* trophozoites.

To determine the minimum percentage of formalin that could be used to kill *B. mandrillaris* trophozoites, the amoebae were treated with different percentages of formalin and the viability was checked by incubation on fresh MA104 monkey kidney cells monolayers. Visualisation of MA104 monolayers within 24 hours after the addition of *B. mandrillaris* trophozoites that had been treated with 0.5% (v/v), 1% (v/v) and 5% (v/v) formalin showed detaching of the MA104 cells from the flask's surface. Therefore, a minor change was made by washing the formalin-treated *B. mandrillaris* trophozoites with DPBS prior to inoculation onto MA104 monolayers. With this modification, the MA104 monolayer remained attached to the flask surface and none of the flasks containing *B. mandrillaris* trophozoites treated with 0.5% (v/v) formalin (Figure 6.3A), 1% (v/v) formalin (Figure 6.3B) and 5% (v/v) formalin (Figure 6.3C) showed any clearing of MA104 cells after 10 days on incubation. Similar observation was seen in the negative control flask (Figure 6.3D). The positive control flask (Figure 6.3E), using untreated *B. mandrillaris* trophozoites showed abundant growth of the amoebae with clearing of monolayer cells after 7 days. As 0.5% (v/v) formalin was sufficient to kill the *B. mandrillaris* amoebae, this percentage was used for the production of *B. mandrillaris* polyclonal and monoclonal antibodies.

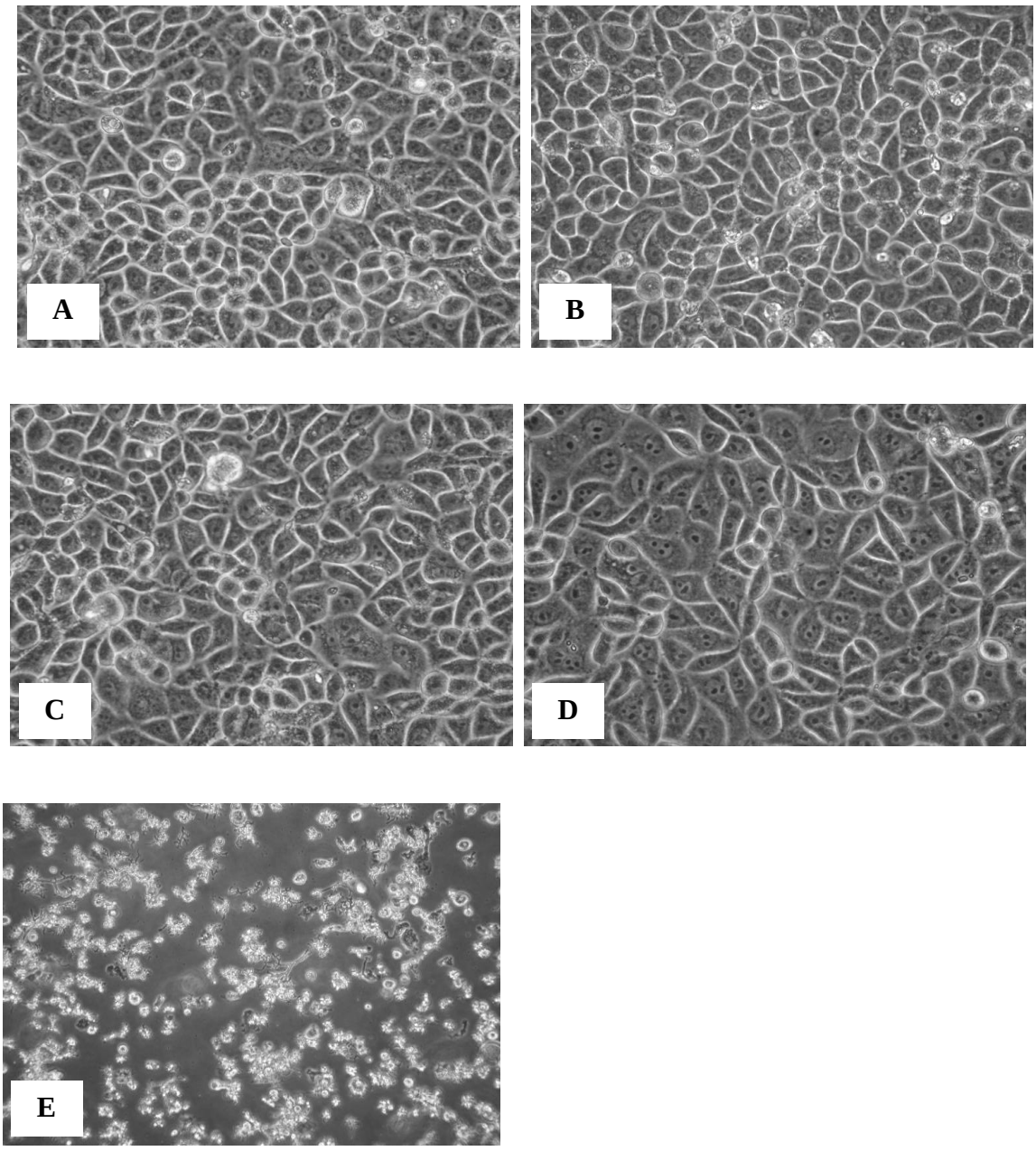
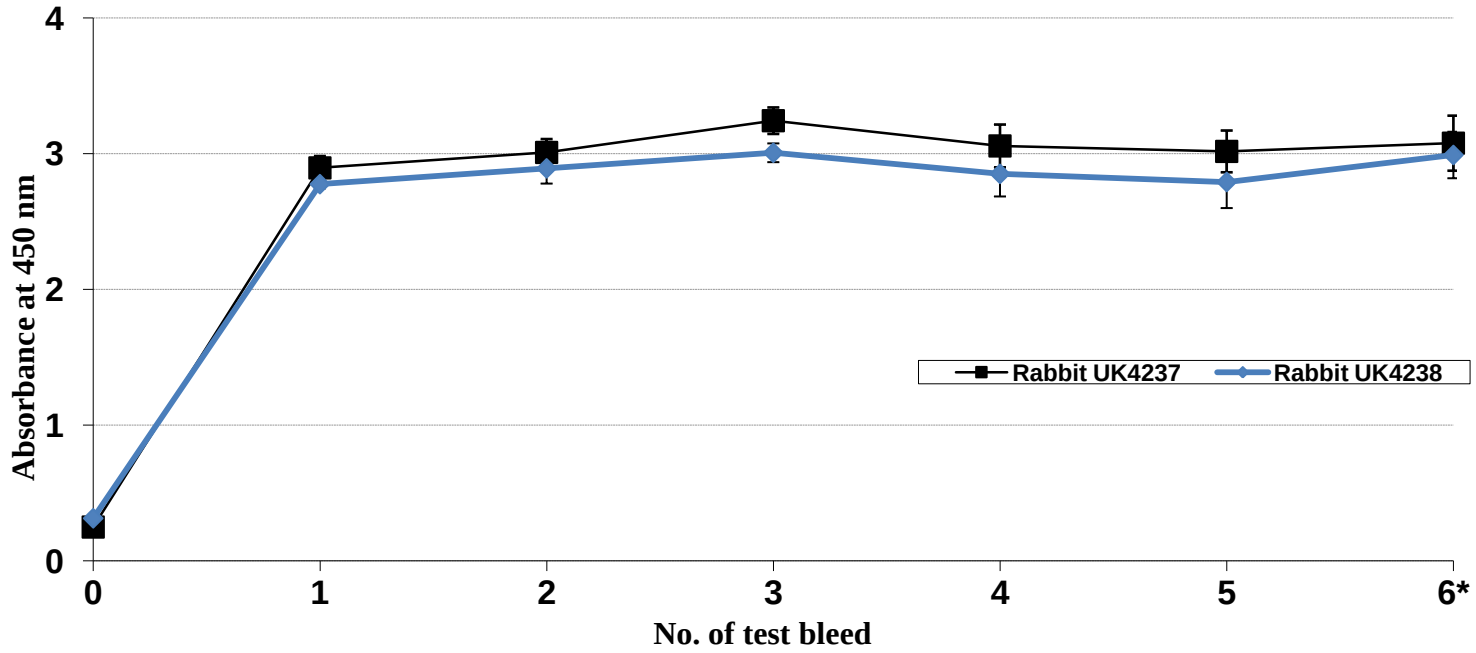


Figure 6.3 Monkey kidney cells monolayer (MA104) inoculated with *B. mandrillaris* trophozoites treated with various percentages of formalin. A: 0.5% (v/v) formalin-treated *B. mandrillaris* (400×); B: 1% (v/v) formalin-treated *B. mandrillaris* (400×); C: 5% (v/v) formalin-treated *B. mandrillaris* (400×); D: negative control (1 ml of DPBS, 400×); E: positive control (untreated *B. mandrillaris*, 200×)

6.2.3 Determination of *B. mandrillaris* antibody levels in rabbits following immunisations with formalin-treated *B. mandrillaris*

Both rabbits (UK4237 and UK4238) were confirmed to be *B. mandrillaris* antibody-negative, based on ELISA results using sera of the pre-immune test bleed taken prior to the scheduled immunisations. Five test bleeds were subsequently done prior to harvest bleed and sera were tested by ELISA against sonicated *B. mandrillaris* to determine increase in *B. mandrillaris* antibody levels in the rabbits. Reactivity of sera from test bleed 1 of rabbits UK4237 and UK4238 following two initial immunisations showed increased absorbance ($p<0.0001$) or *B. mandrillaris* antibody levels (Figure 6.4). Further immunisations to both rabbits resulted in no further significant increase ($p>0.05$) in absorbance readings for sera from test bleed 2 to test bleed 5. Both rabbits were then kept for another 2 weeks before a harvest bleed was performed. No significant changes ($p>0.05$) in the absorbance was seen with sera from the harvest bleed was compared to the test bleed 5 (Figure 6.4, test bleed 6).

Figure 6.4 *B. mandrillaris* antibody levels in rabbits UK4237 and UK4238 following immunisations with formalin-killed *B. mandrillaris* trophozoites



* = Harvest bleed. Results are mean of experiment performed in triplicates. Error bars represent SEM

6.2.4 Specificity of large-scale polyclonal antibodies against *B. mandrillaris*

In determining the specificity of large-scale polyclonal antibodies against *B. mandrillaris*, three different assays (ELISA, immunoperoxidase and immunofluorescence) were performed with various free-living amoebae. These were *Balamuthia mandrillaris* (ATCC 50209), *Acanthamoeba castellanii* (ATCC 50370) and *Naegleria gruberi* (ATCC 30224). Sera from both rabbits were purified and concentrated prior use in the three assays. The reactivity of the concentrated sera against *B. mandrillaris* was checked by ELISA prior to the specificity assays and results are shown in Figure 6.5. Concentrated sera from both rabbits, UK4237 and UK4238, exhibited the same intensity of colour reaction at dilutions 1:50 to 1:102,400 when reacted against sonicated *B. mandrillaris* trophozoites. Therefore, the subsequent specificity assays were only performed using concentrated polyclonal antibody from rabbit UK4237.

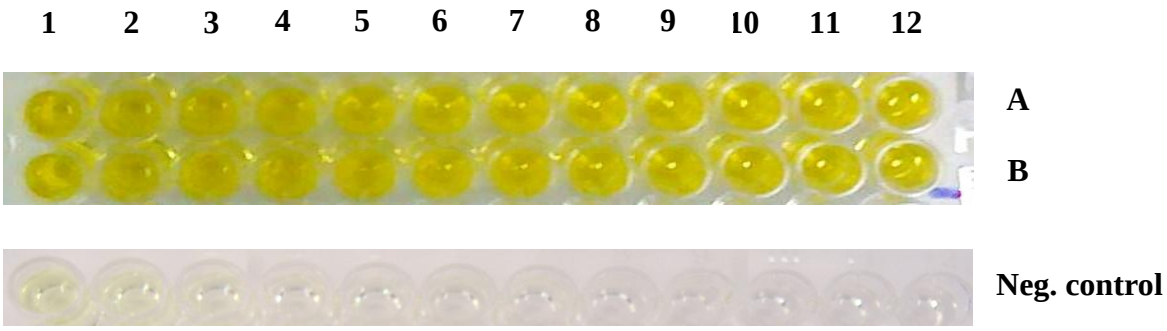


Figure 6.5 Reactivity of purified and concentrated polyclonal antibodies of rabbits UK4237 (A) and UK4238 (B) against sonicated *B. mandrillaris* by ELISA. Well 1: 1/50; Well 2: 1/100; Well 3: 1:200; Well 4: 1:400; Well 5: 1/800; Well 6: 1/1600; Well 7: 1/3200; Well 8: 1/6400; Well 9: 1/12,800; Well 10: 1/25,600; Well 11: 1/51,200; Well 12: 1/102,400.

To determine the specificity of the purified and concentrated polyclonal antibodies, an ELISA was performed against different free-living amoebae, using only a single dilution (1:3200) of polyclonal antibody UK4237 but with two dilutions of secondary antibody (1:10,000 and 1:25,000). As shown in Table 6.1, the highest antigen-antibody reaction was seen in wells coated with *B. mandrillaris* trophozoites, with an $A_{450\ nm}$ of 2.97 (1:10,000) and 1.16 (1:25,000). Cross-reactivity was seen against *Acanthamoeba castellanii*, with A_{450nm} of 1.78 (1:10,000) and 0.79 (1:25,000). When the polyclonal antibody UK4237 were reacted with wells coated with *Naegleria gruberi*, a slight cross-reactivity was detected, with A_{450nm} of 0.54 and 0.25 for secondary antibody dilutions of 1:10,000 and 1:25,000 respectively. Controls using pre-bleed serum showed A_{450nm} of 0.25 (1:10,000) and 0.11 (1:25,000). Since higher A_{450nm} readings were obtained using secondary antibody at a dilution of 1:10,000 than at 1:25,000, the following immunoperoxidase assays, which also involved the use of the same secondary antibody, were only performed at dilution of 1:10,000.

Table 6.1 Test of specificity of UK4237 polyclonal antibody against different free-living amoebae by ELISA

Source of antigen	**A_{450nm}**	
	Secondary antibody dilution	
	1:10,000	**1:25,000**
Balamuthia mandrillaris	2.97	1.16
Acanthamoeba castellanii	1.78	0.79
Naegleria gruberi	0.54	0.25
Negative control	0.25	0.11

The optimisation of immunoperoxidase and immunofluorescence assays was initially performed using Teflon slides, as suggested in the literature. Attempts to adhere the *B. mandrillaris* trophozoites to the wells of the Teflon slides were unsuccessful. This was in contrast to the experience with *A. castellanii* trophozoites. In addition, if the areas surrounding the wells were not properly dried following the washing steps, the contents of different wells could easily get mixed. As an alternative, both the assays were performed using Maxisorb 96-well plates (Nunc, Thermo Scientific). Three dilutions (1:2,500, 1:25,000 and 1:250,000) of purified and concentrated UK4237 polyclonal antibody were tested in the immunoperoxidase assay. A strong blue colouration at the cell membrane was seen when a dilution of 1:2500 was tested against *B. mandrillaris* trophozoites (Figure 6.6A). No colouration was seen for controls performed using only the secondary antibody plus substrate (Figure 6.6B) or substrate alone (Figure 6.6C). *Acanthamoeba* trophozoites slightly reacted with the same dilution of UK4237 polyclonal antibody staining faint blue. This indicated a slight cross-reactivity (Figure 6.7A). No colouration was seen for the *Acanthamoeba* controls which were done by including only the secondary antibody plus substrate (Figure 6.7B) or substrate alone (Figure 6.7C). With *Naegleria gruberi* trophozoites no colour was seen when a 1:2500 dilution of polyclonal antibody UK4237 was used (Figure 6.8A). Similar negative reactions were also shown with controls done with secondary antibody plus substrate (Figure 6.8B) or substrate alone (Figure 6.8C). Subsequent experiments done against those three amoebae using polyclonal antibody UK4237 at dilutions of 1:25,000 and 1:250,000 showed negative results (no colouration).

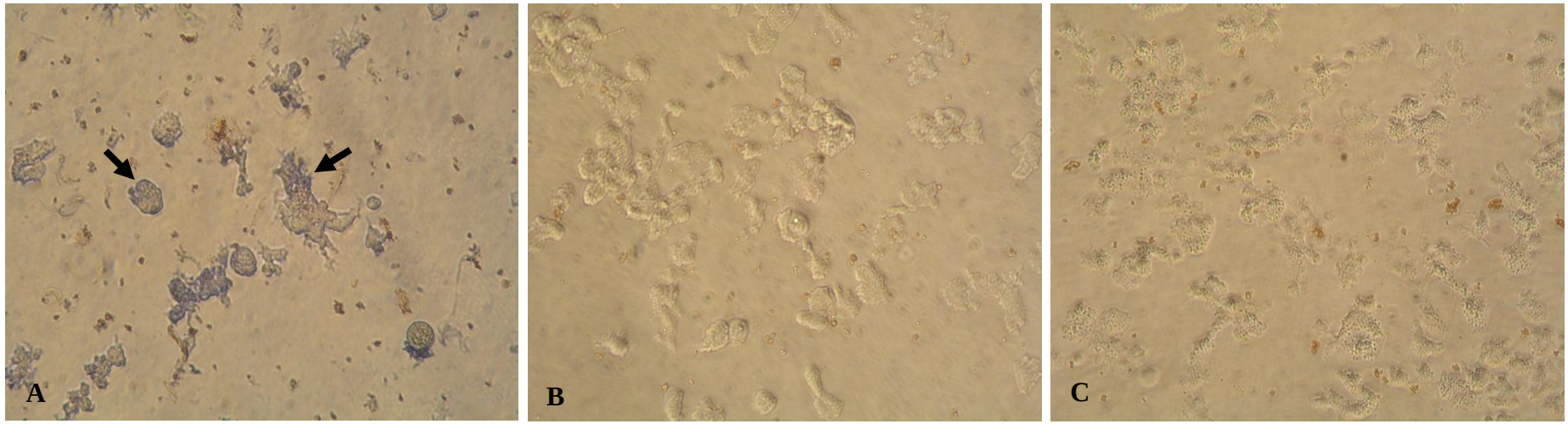


Figure 6.6 Specificity of purified and concentrated polyclonal antibody UK4237 (1:2,500) against *B. mandrillaris* (ATCC 50209) trophozoites by immunoperoxidase assay. Reactivity of the polyclonal antibody with the trophozoites showed a strong positive reaction as shown by dark blue colouration (arrows) at the cell membrane (A). Controls wells that were added with secondary antibody plus substrate (B) or substate alone (C) showed negative results (no colouration). All images at 400×

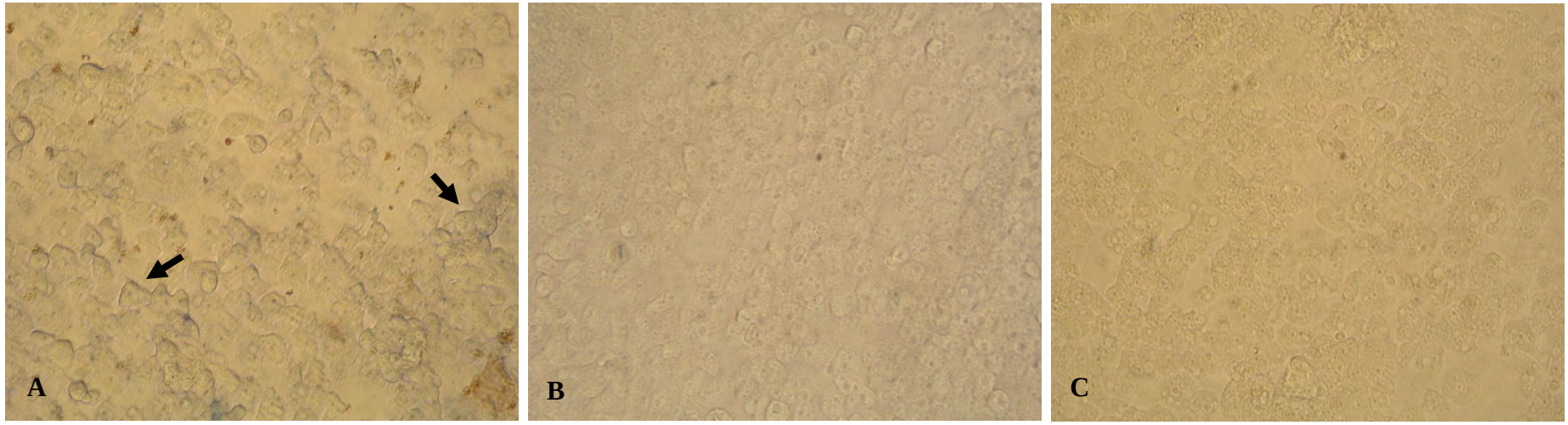


Figure 6.7 Specificity of purified and concentrated polyclonal antibody UK4237 (1:2,500) against *Acanthamoeba castellanii (*ATCC 50370) trophozoites by immunoperoxidase assay. A slight cross-reactivity was shown by faint blue staining (arrows) at the cell membrane of the trophozoites (A). Controls wells that were added with secondary antibody plus substrate (B) or substate alone (C) showed negative results (no colouration). All images at 400×

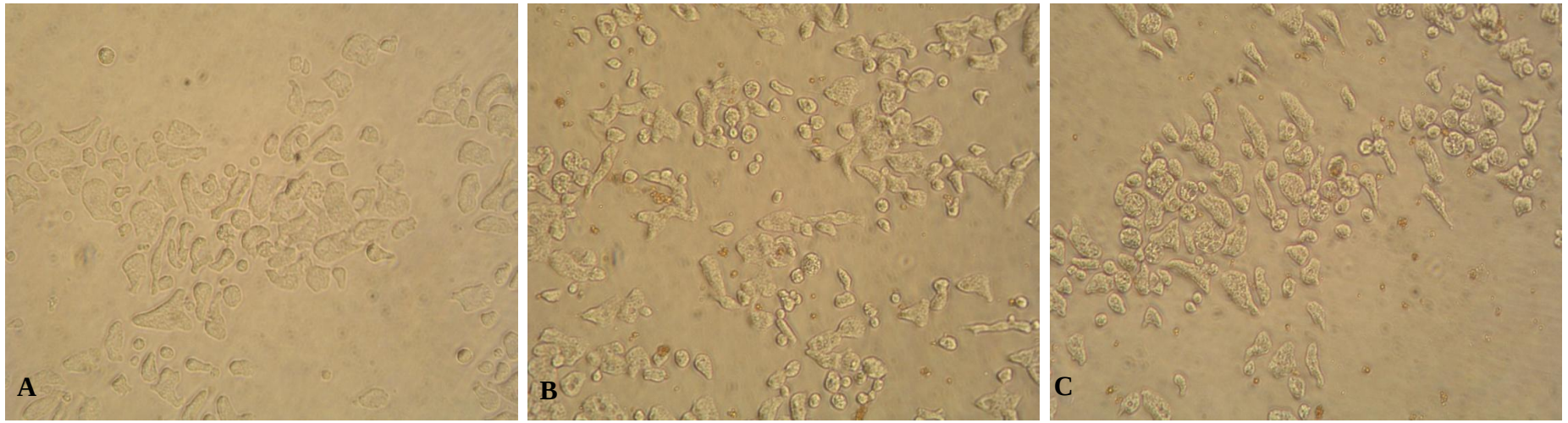


Figure 6.8 Specificity of purified and concentrated polyclonal antibody UK4237 (1:2,500) against *Naegleria gruberi (*ATCC 30224) trophozoites by immunoperoxidase assay. Negative reaction (no colouration) was seen when the polyclonal antibody was reacted with the trophozoites. Similar reactions were also seen in control wells that were added with secondary antibody plus substrate (B) or substate alone (C) All images at 400×

The results for the immunofluorescence assay are summarised in Table 6.2. A strong or punctate fluorescence (3+) at the cell membrane of *B. mandrillaris* trophozoites was seen when purified and concentrated polyclonal antibody UK4237 was used at dilutions of 1:50 (Figures 6.9A and 6.9B) to 1:3200. The intensity of the fluorescent started to drop at a dilution of 1:6400 (2+) and showed a weak fluorescence (+) with polyclonal antibody dilution of 1:25,600. No fluorescence was seen with the negative control (Figure 6.9C and Figure 6.9D).

When the purified and concentrated polyclonal antibody UK4237 were reacted with *A. castellanii* trophozoites, a 3+ fluorescence was seen at dilution of 1:50 (Figure 6.10A and Figure 6.10B). A drop of fluorescence intensity started to occur with dilution of 1:100 (2+) and a very weak fluorescence (±) could be observed with polyclonal antibody at a dilutions of 1:800 (Figure 6.10C and Figure 6.10D) and 1:1600. No fluorescence was seen with dilutions of 1:3200 to 1:25600. *A. castelanii* negative control that had only secondary antibody showed no fluorescence (Figure 6.10E and Figure 6.10F).

For *N. gruberi*, a weak fluorescence (1+) was observed when the polyclonal antibody with dilutions of 1:50 (Figure 6.11A and Figure 6.11B) to 1:200 were used. Subsequent dilutions from 1:400 (Figure 6.11C and Figure 6.11D) to 1:1600 yielded a very weak fluorescence, as indicated by the '±' sign. Assay with polyclonal antibody dilutions of 1:3200 to 1:25600 showed no fluorescence. Similar result was also shown by the negative control (Figure 6.11E and Figure 6.11F).

Table 6.2 Specificity of purified and concentrated polyclonal antibody UK4237 against free-living amoebae in immunofluorescence assay

Dilution of polyclonal antibody	**Degree of fluorescence against free-living amoeba with polyclonal antibody UK4237**		
	B. mandrillaris	***A. castellanii***	***N. gruberi***
1:50	3+	3+	+
1:100	3+	2+	+
1:200	3+	+	+
1:400	3+	+	±
1:800	3+	±	±
1:1600	3+	±	±
1:3200	3+	neg	neg
1:6400	2+	neg	neg
1:12800	2+	neg	neg
1:25600	+	neg	neg
Control	neg	neg	neg

Degree of fluorescence: 3+ = strong; 2+ = moderate; + = weak; ± = very weak

neg = no fluorescence

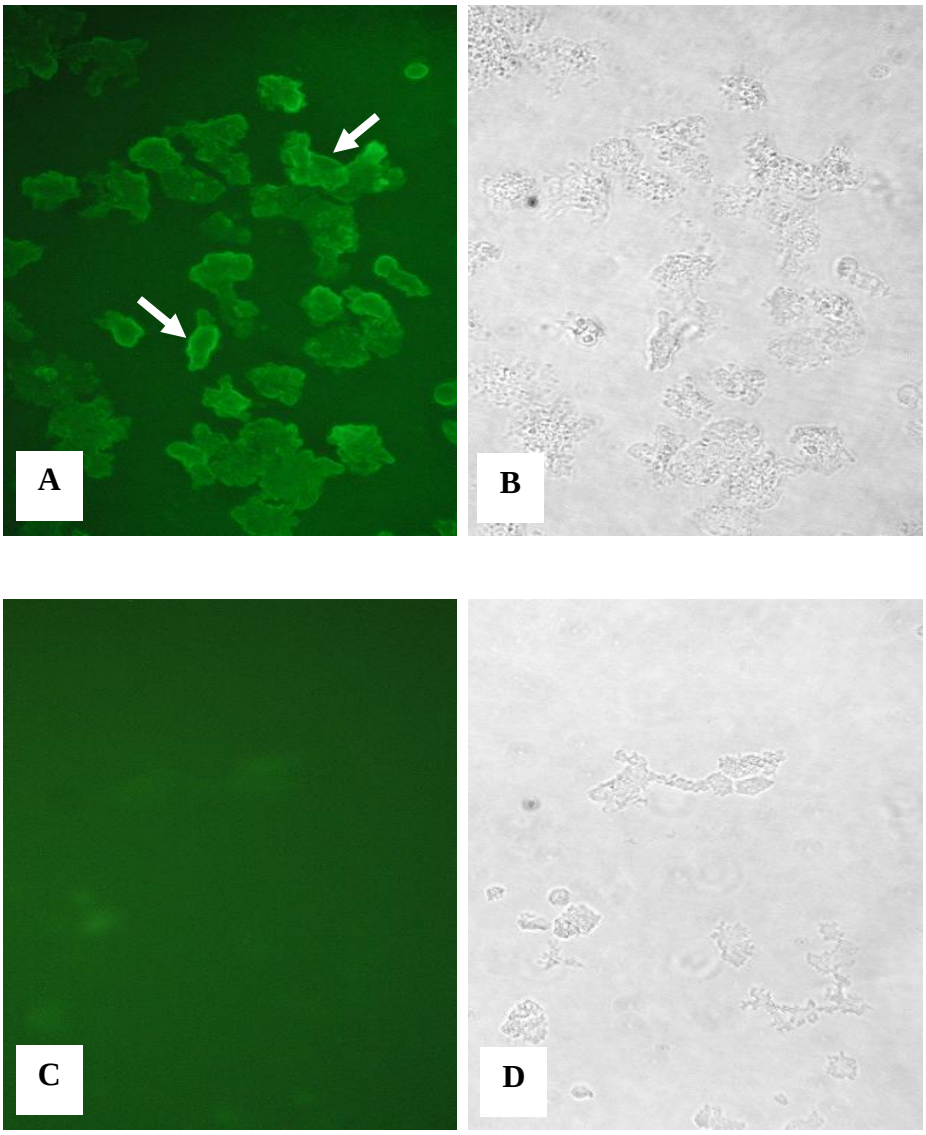


Figure 6.9 Specificity of purified and concentrated polyclonal antibody UK 4237 (1:50) against *Balamuthia mandrillaris (*ATCC 50209) trophozoites in an immunofluorescence assay. (A) A strong fluorescence (3+) was observed at the cell membrane of the trophozoites (arrows, 400×). (C) No fluorescence was seen in control wells with only FITC-secondary antibody added (200×). (B) and (D) show corresponding phase-contrast images

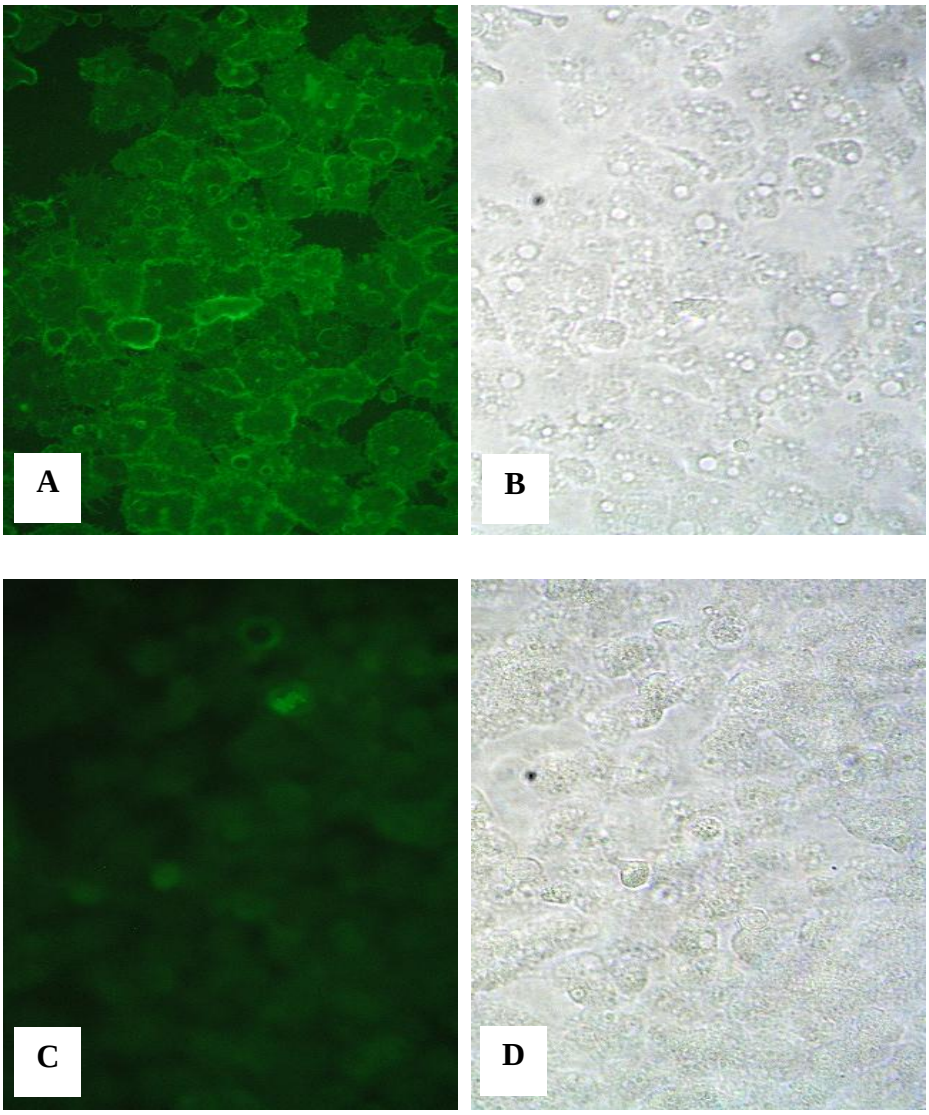


Figure 6.10 Specificity of purified and concentrated polyclonal antibody UK4237 against *Acanthamoeba castellanii (*ATCC 50370) trophozoites in an immunofluorescence assay. (A) A strong fluorescence (3+) especially at the cell membrane was seen when the polyclonal antibody at a dilution of 1:50 was used. (C) Very weak fluorescence (±) was seen with a dilution of polyclonal antibody of 1:800. (B) and (D) show corresponding phase-contrast images. All images at 400×

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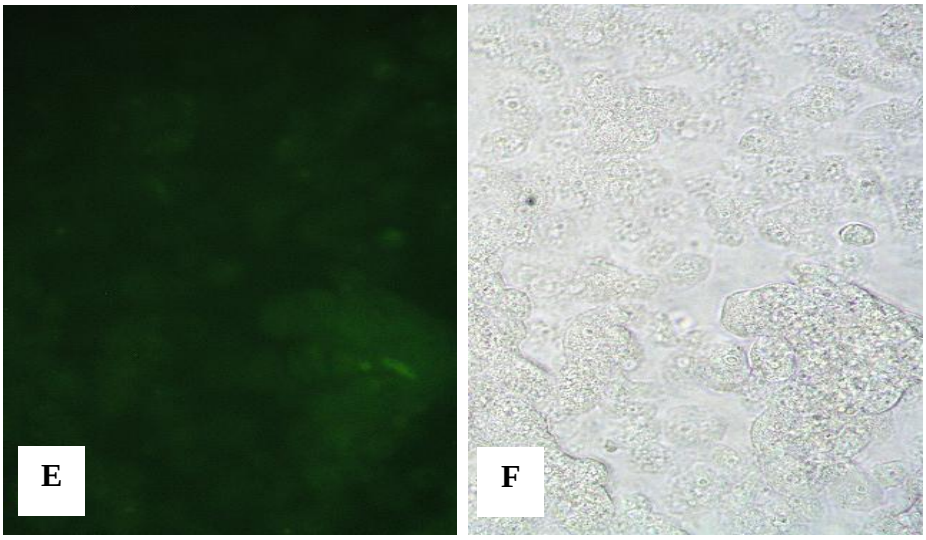


(E) Control wells with only FITC-secondary antibody added showed no fluorescence.

(F) shows corresponding phase-contrast image. All images at 400×

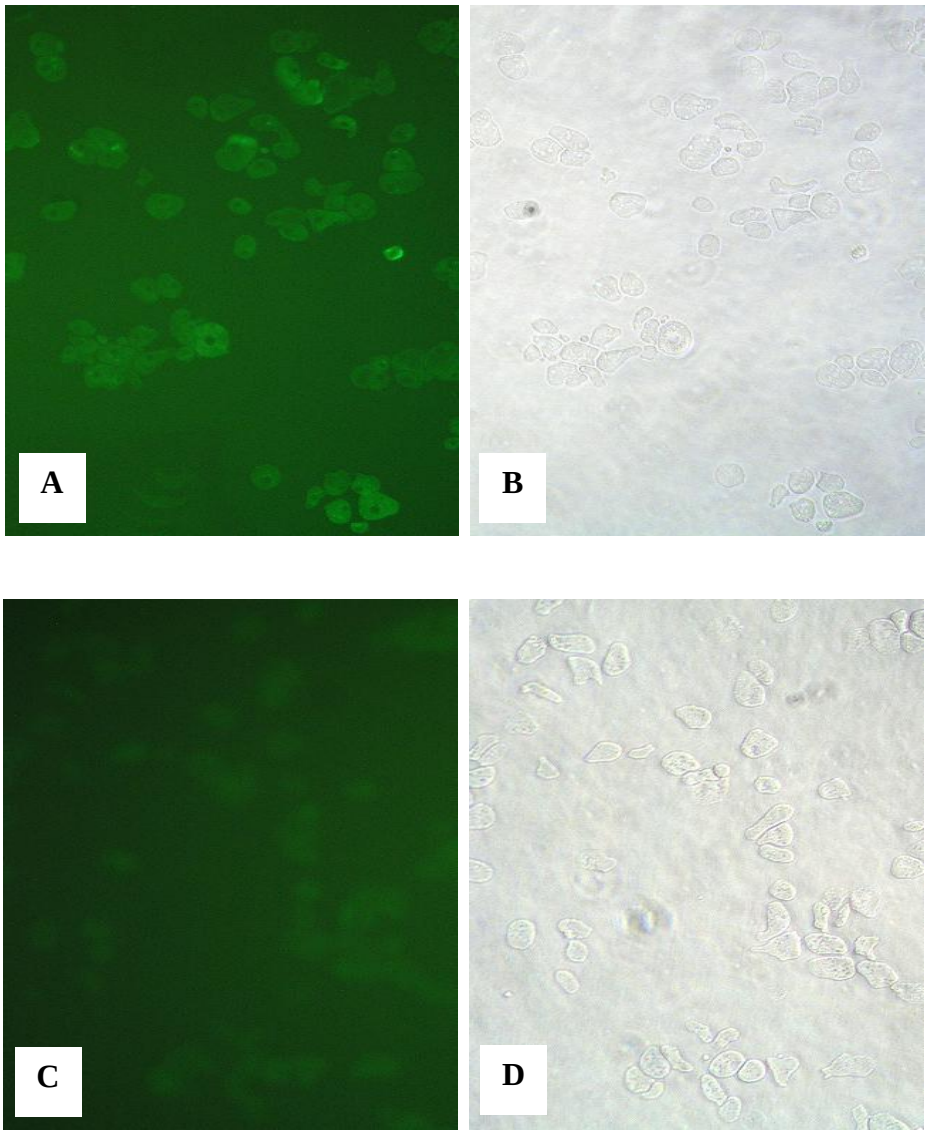
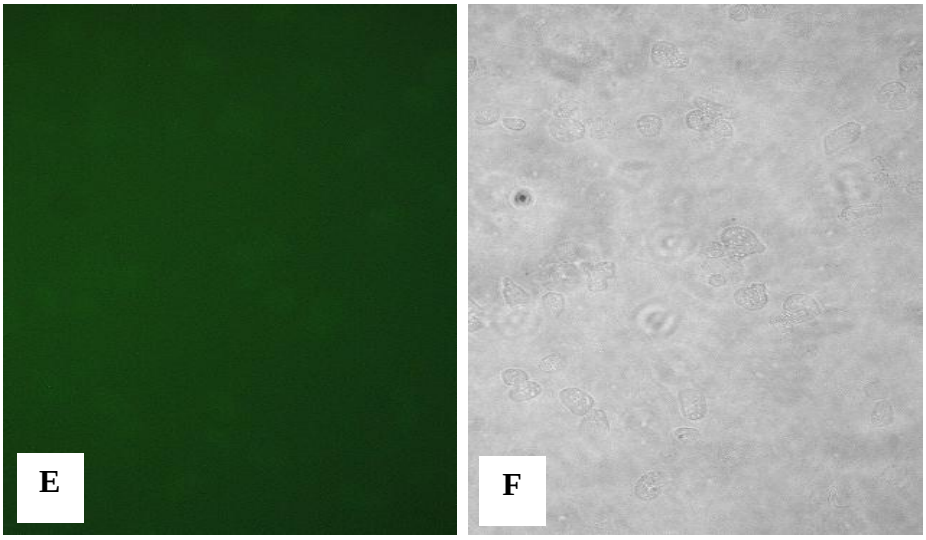


Figure 6.11 Specificity of purified and concentrated polyclonal antibody UK4237 against *Naegleria gruberi* (ATCC 30224) trophozoites in an immunofluorescence assay. (A) A weak fluorescence (+) showed with polyclonal antibody at a dilution of 1:50. (C) *N. gruberi* trophozoites showed a very weak fluorescence (±) with polyclonal antibody dilution of 1:400. All images at 400×

Continued:



(E) No fluorescence was seen in control wells with only FITC-secondary antibody added. (F) shows corresponding phase-contrast images. All images at 400×

6.2.5 Production of anti-*B. mandrillaris* monoclonal antibodies

The production of monoclonal antibodies (MAb) against *B. mandrillaris* was in collaboration with Cambridge Reasearch Biochemicals (CRB). The project involved 4 phases: immunisation, fusion, cloning and expansion. The antibody levels at each phase were checked by ELISA tests performed at CRB and were reconfirmed with second ELISA tests done at the University of Leicester. Antigens were prepared at the University of Leicester.

During the 1st phase (immunisation) all ELISA results from CRB were given as titre. Initially, 5-fold dilutions of sera from immunised mice were prepared and the reactivity with whole *B.* mandrillaris trophozoites was measured by absorbance at 415 nm. A graph was plotted and the titre that gave 50% of the maximum achieved response (absorbance) was determined. The descriptions of the titres that gave 50% of the maximum absorbance are given in Table 6.3.

Table 6.3 Description of titre giving 50% maximum absorbance

Titre	**Description**
<1000	Very low
1,000 to 5,000	Low
5,000 to 10,000	Average
>20,000	High

All five immunised mice (UK4241, UK4242, UK4243, UK4244 and UK4245) were confirmed to be free from anti-*B. mandrillaris* antibodies by ELISA prior to immunisations. Subsequently, mice were immunised for five times and three test bleeds

were performed between the scheduled immunisation (see Section 2.24 for details). The ELISA for sera of test bleed 1 and 2 were performed against whole-cell (formalin-killed) *B. mandrillaris* trophozoites as the antigen. Sera from test bleed 1 of all mice showed very low titre, which ranged from less than 100 to 470 (Table 6.4). For sera of test bleed 2, a slight increase (140 to 2,570) in the titre was seen for all the mice except mouse UK 4241 (Table 6.4). As the results were still interpreted as very low titre based on the reference in Table 6.3, it was decided to perform ELISA with test bleed 3 sera using plates coated with whole and lysed (sonicated) *B. mandrillaris* trophozoites to check whether different antigen preparations could affect the antigen-antibody reaction. Table 6.5 shows that the titre results for test bleed 3 were better when whole cells were used rather than the lysed cells. The highest titre with both types of cells preparation was seen with serum from mouse UK4245 with a titre of 70,500 and 14,000 respectively.

Table 6.4 The titre for test-bleeds 1 and 2 sera against whole *B. mandrillaris* trophozoites

Animal ID	**Titre (whole cells)**	
	Test bleed 1	**Test bleed 2**
UK4241	140	140
UK4242	164	590
UK4243	470	1655
UK4244	230	2570
UK4245	<100	400

Table 6.5 The titre for test bleed 3 sera against whole and lysed *B. mandrillaris* trophozoites

Animal ID	**Titre (whole cells)**	**Titre (lysed cells)**
UK4241	22,500	3,500
UK4242	15,000	1,700
UK4243	16,000	2,300
UK4244	14,000	6,700
UK4245	70,500	14,000

An aliquot of the UK 4245 serum from test bleed 3 was re-assayed by ELISA using whole and lysed *B. mandrillaris* trophozoites at the University of Leicester. As shown in Figure 6.12, ELISA performed using whole (Rows A and B) and lysed (Rows C and D) *B. mandrillaris* trophozoites showed no difference in the colour intensity produced. Very strong blue was seen with dilutions of 1:50 (well 1) to 1:6400 (well 8), while subsequent dilutions of serum gave lighter blue colouration. The intensity of the colour was much easier to be differentiated when it was blue (before the addition of H_2SO_4) rather than when the colour was yellow (after the addition of H_2SO_4). In line with the results of titre given by the CRB, mouse UK4245 was chosen for phase II (fusion of spleen cells with immortalised cell line). Subsequent ELISA done at CRB and University of Leicester were performed using whole and lysed *B. mandrillaris* trophozoites respectively.

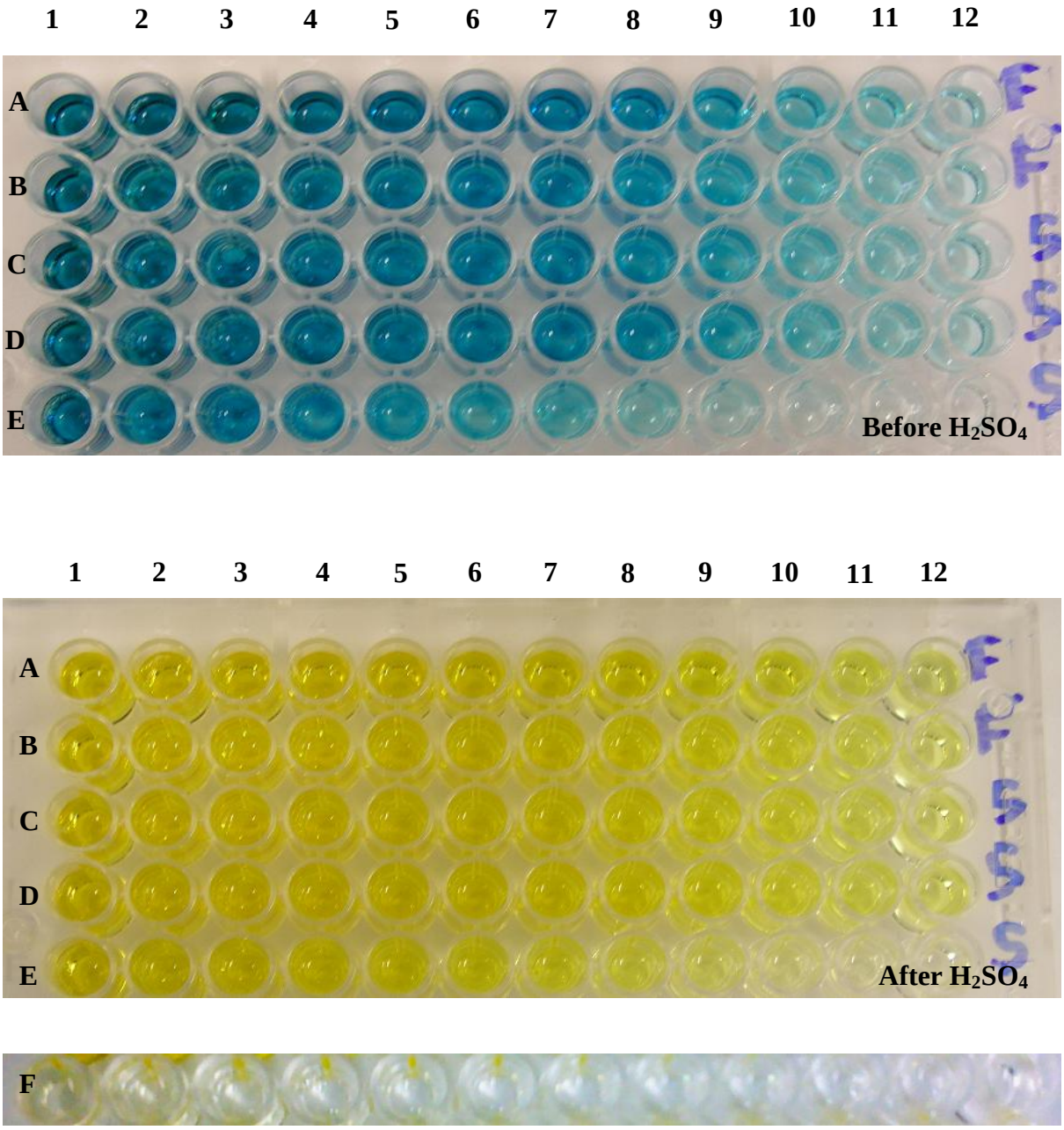


Figure 6.12 Reactivity of serum from test bleed 3 of mouse UK4245 in an ELISA with whole (formalin-killed) and lysed (sonicated) *B. mandrillaris*. This experiment was done at University of Leicester. Rows A and B: reactivity of UK4245 serum with formalin-killed *B. mandrillaris*; Rows C and D: reactivity of 4245 serum with sonicated *B. mandrillaris*. Row E: reactivity of positive control serum with sonicated *B. mandrillaris*. Row F: reactivity of negative control serum with sonicated *B. mandrillaris*. Well 1: 1/50; Well 2: 1/100; Well 3: 1/200; Well 4: 1/400; Well 5: 1/800; Well 6: 1/1600; Well 7: 1/3200; Well 8: 1/6400; Well 9: 1/12,800; Well 10: 1/25,600; Well 11: 1/51,200; Well 12: 1/102,400

A total of 31 culture medium samples were received after the 2nd (fusion) phase. The ELISA done at CRB showed that only 6 clones (C7, C5, C10, C128, C49 and C113) gave an A_{415nm} above 0.1, while the A_{415nm} for the other 26 clones was below 0.05 (Table 6.6). Therefore, further ELISAs test performed at the University of Leicester were only against the 6 clones giving an $A_{415nm} > 0.1$. At the University of Leicester, ELISA results were interpreted based on the intensity of the colour reaction produced. The strongest colour reaction was obtained with clone C7 followed by C10, C5 and C113. No reaction was seen with C128 and C49 (Figure 6.13).

Because CRB allowed the client to choose only 3 clones for the 3rd phase (cloning), clones C7, C10 and C5 were chosen to be used for cloning purposes. Following the cloning process, another 17 culture medium samples from subclones of C7 (12 samples) and C10 (5 samples) were received from the CRB for reassayed prior to the 4th phase (expansion). The CRB's ELISA results for the 3rd phase are summarised in Table 6.7. No subclones were received from clone C5 because of the poor absorbance in ELISA (less than 0.03, results not shown). The 17 samples were reassayed at the University of Leicester and all but SC53 of C10 showed strong antigen-antibody reaction based on the intensity of the colour produced in ELISA tests as shown in Figure 6.14. A single subclone of each C7 (SC62) and C10 (SC14) was chosen for long-term cells expansion and they were tested for their specificity against *B. mandrillaris*. Both subclones (SC62 and SC14) were identified as, producing IgG1, with a Hycult monoclonal antibody isotyping kit.

Table 6.6 Absorbance at 415 nm in ELISA with supernatant of clones of UK4245 after 2nd phase (fusion)*

Clones from UK4245	**Absorbance $_{415nm}$**	**Clones from UK4245**	**Absorbance $_{415nm}$**
C7	0.597	132	0.032
C5	0.596	17	0.032
C10	0.560	41	0.031
C128	0.315	3	0.031
C49	0.217	15	0.031
C113	0.103	2	0.031
C50	0.052	45	0.030
C127	0.046	42	0.030
C4	0.040	26	0.030
C55	0.037	32	0.029
C155	0.036	76	0.029
C23	0.035	112	0.029
C19	0.035	158	0.029
C31	0.034	13	0.028
C25	0.033	54	0.027
C29	0.033		

Clones chosen for second ELISA test

* = done by Cambridge Research Biochemicals

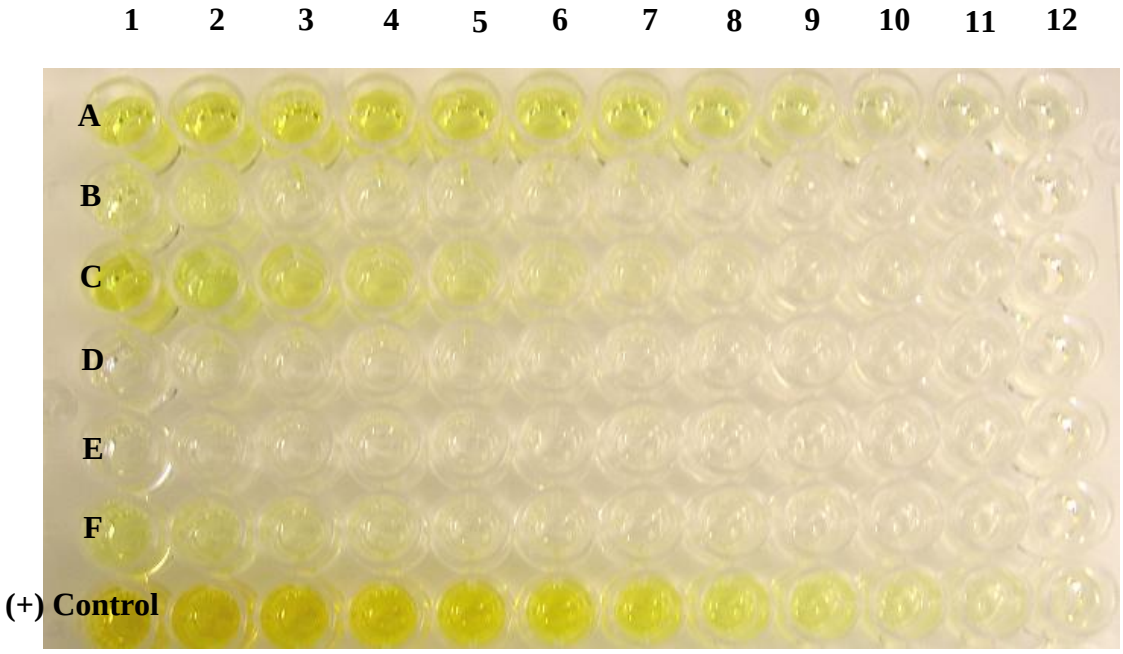


Figure 6.13 ELISA results for clones C7 (A), C5 (B), C10 (C), C128 (D), C49 (E) and C113 (F) derived from mouse UK4245 after 2nd phase (fusion). These were done at the University of Leicester. Well 1: 1/50; Well 2: 1/100; Well 3: 1/200; Well 4: 1/400; Well 5: 1/800; Well 6: 1/1600; Well 7: 1/3200; Well 8: 1/6400; Well 9: 1/12,800; Well 10: 1/25,600; Well 11: 1/51,200; Well 12: 1/102,400

Table 6.7 Absorbance at 415 nm in ELISA with supernatant of subclones of C7 and C10 of UK4245 after 3rd phase (cloning)*

Subclones of Clone 7	**Absorbance $_{415nm}$**
SC154	1.492
SC28	1.581
SC62	1.589
SC167	1.395
SC15	1.421
SC168	1.252
SC108	1.437
SC29	1.577
SC23	1.025
SC37	1.056
SC53	1.03
SC132	0.954
Subclones of Clone 10	**Absorbance $_{415nm}$**
SC151	1.573
SC14	1.716
SC5	1.568
SC178	1.436
SC53	0.341

* done by Cambridge Research Biochemicals

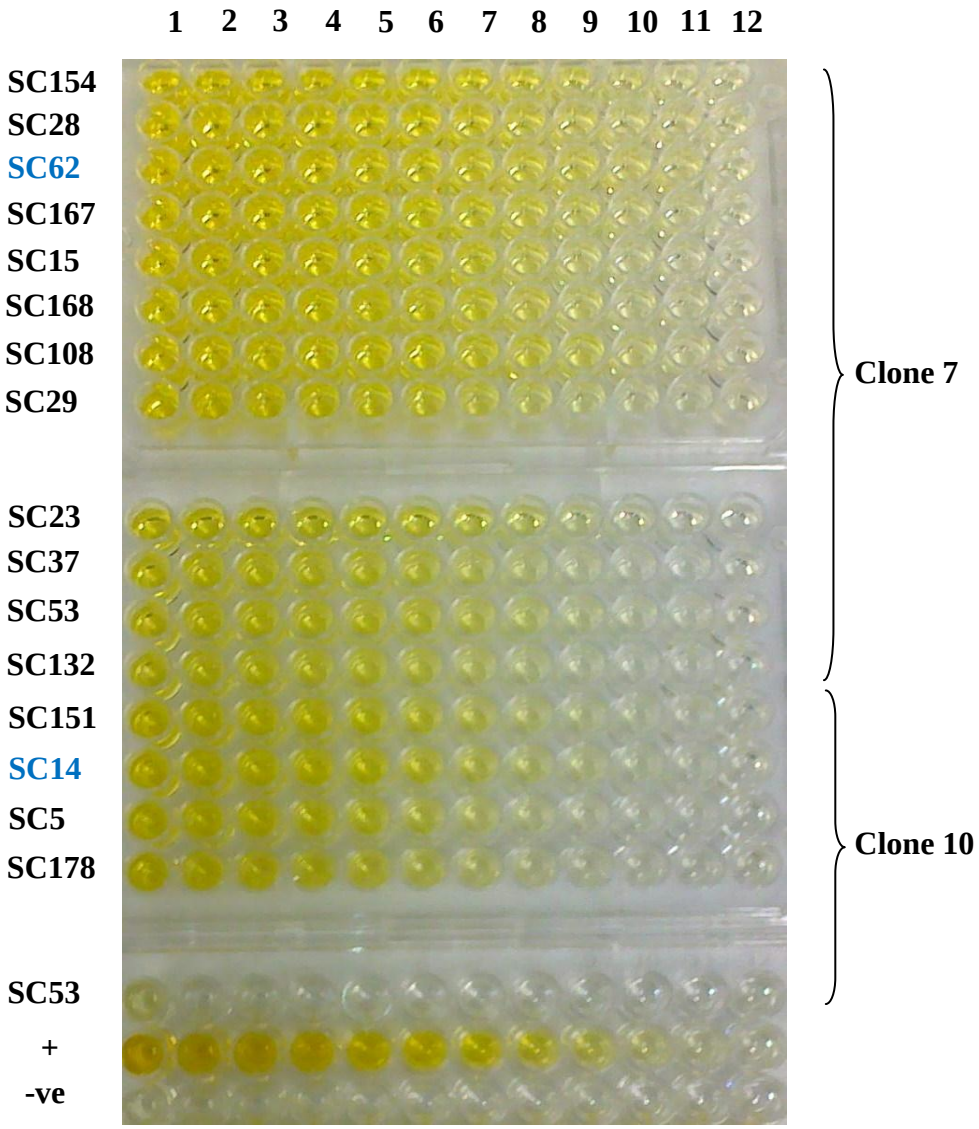


Figure 6.14 ELISA results using supernatant from subclones of Clone 7 and Clone 10 of UK4245 after 3rd phase (cloning). These were done at the University of Leicester. Well 1: 1/50; Well 2: 1/100; Well 3: 1/200; Well 4: 1/400; Well 5: 1/800; Well 6: 1/1600; Well 7: 1/3200; Well 8: 1/6400; Well 9: 1/12800; Well 10: 1/25,600; Well 11: 1/51,200; Well 12: 1/102,400. SC: subclone; +: positive control; - ve: negative control. Fonts in blue indicate subclones chosen for cells expansion

6.2.6 Specificity of monoclonal antibodies

Two approaches (immunofluorescence assay and ELISA) were used to determine the specificity of subclones SC62 and SC14 against *B. mandrillaris*. These two subclones gave monoclonal antibodies MAbSC62 and MAbSC14 respectively. Out of three free-living amoebae (*B. mandrillaris*, *A. castellanii*, *N. gruberi*) tested, only *B. mandrillaris* showed the bright green fluorescence indicative of a positive reaction. The results for immunofluorescence assay are summarised in Table 6.8. The strongest reaction with highest fluorescence intensity (3+) was seen with a dilution of 1:50 for both MAbSC62 (Figure 6.15A) and MAbSC14 (Figure 6.15C). A 2+ reaction was seen when the monoclonal antibodies were used at a dilution of 1:100. The degree of fluorescence was much less with dilutions 1:400 and 1:800. The negative control with only *B. mandrillaris* trophozoites and the secondary antibody anti-mouse IgG-FITC showed no fluorescence (Figure 6.15E). *B. mandrillaris* cysts showed different type of fluorescence giving an orange-green colour (Figure 6.16A) but because the cysts in the control well that were reacted with only anti-mouse IgG-FITC secondary antibody also exhibited the orange-green fluorescence this was considered as autofluorescence (Figure 6.16C). Neither monoclonal antibodies showed fluorescence with *A. castellanii* (Figure 6.17A) and *N. gruberi* (Figure 6.18A). Similar results were also observed from negative control wells with *A. castellanii* (Figure 6.17C) and *N. gruberi* (Figure 6.18C). The respective images for all the reactions using phase-contrast microscope are shown in Figure 6.15B, Figure 6.15D, Figure 6.15F, Figure 6.16B, Figure 6.16D, Figure 6.17B, Figure 6.17D, Figure 6.18B and Figure 6.18D).

For specificity testing using ELISA, as shown in Figure 6.19, only wells coated with *B. mandrillaris* trophozoites showed a positive reaction with monoclonal antibodies MAbSC62 and MAbSC14 at dilutions up to 1:6400 and 1:12,800 respectively. Negative

results were obtained for similar dilutions of both monoclonal antibodies against *A. castellanii* and *N. gruberi* trophozoites (Figure 6.19).

Table 6.8 Specificity of monoclonal antibodies MAbSC62 and MAbSC14 against free-living amoebae tested in immunofluorescence assay

Dilution of MAb	**Degree of fluorescence against free-living amoebae with MAbSC62***		
	B. mandrillaris	***A. castellanii***	***N. gruberi***
1:50	3+	neg	neg
1:100	2+	neg	neg
1:200	+	neg	neg
1:400	±	neg	neg
1:800	±	neg	neg
1:1600	neg	neg	neg
1:3200	neg	neg	neg
1:6400	neg	neg	neg
1:12800	neg	neg	neg
1:25600	neg	neg	neg
Negative control	neg	neg	neg

Degree of fluorescence: 3+ = strong; 2+ = moderate; + = weak; ± very weak

neg = no fluorescence

* = similar results were obtained with MAbSC14

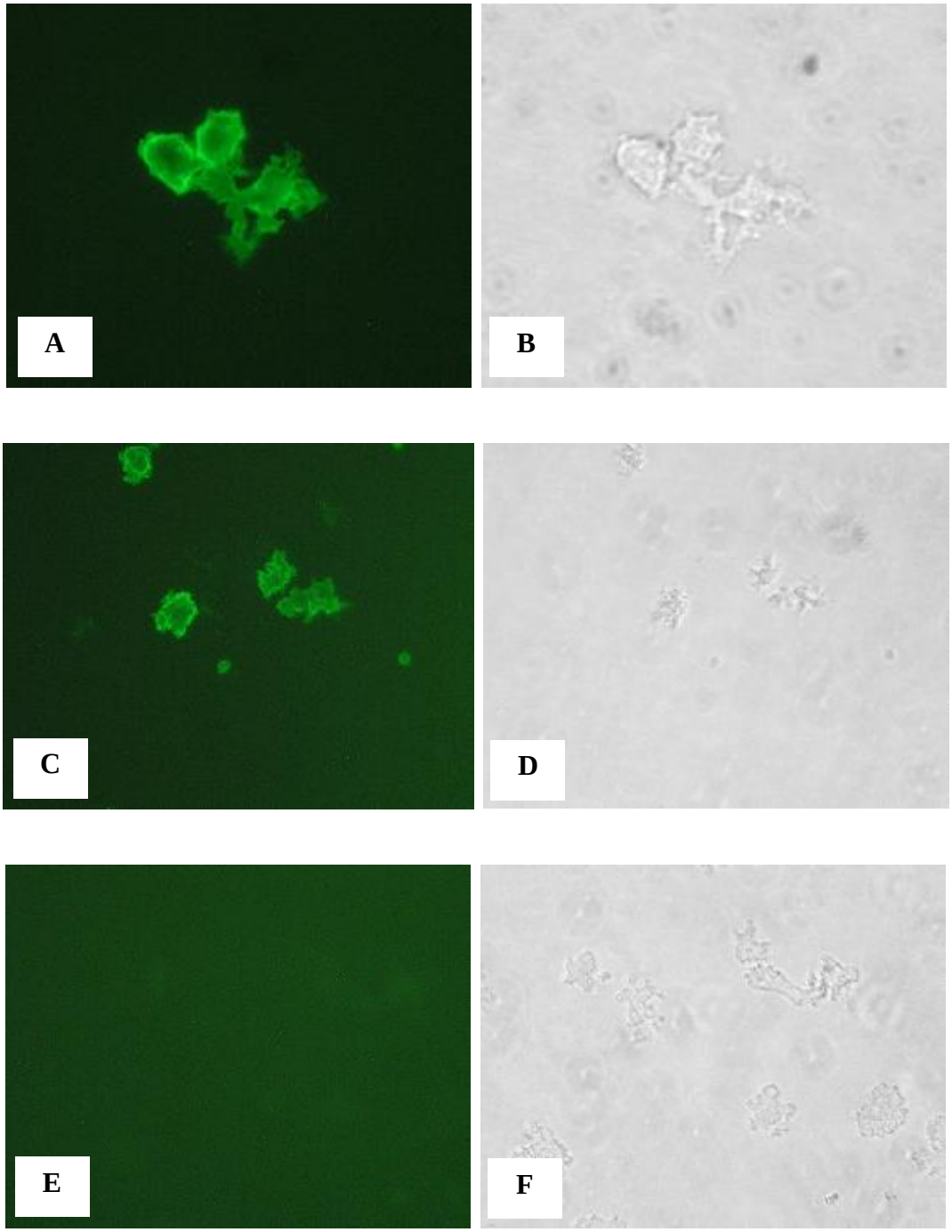


Figure 6.15 Reactivity of MAbSC62 and MAbSC14 (diluted 1:50) against *B. mandrillaris*. A 3+ fluorescence of *B. mandrillaris* trophozoites by MAbSC62 (A, 400X) and MAbSC14 (C, 200X). No fluorescence was seen in the negative control well containing only *B. mandrillaris* and FITC-secondary antibody (E). Respective images under phase contrast (B, D and F)

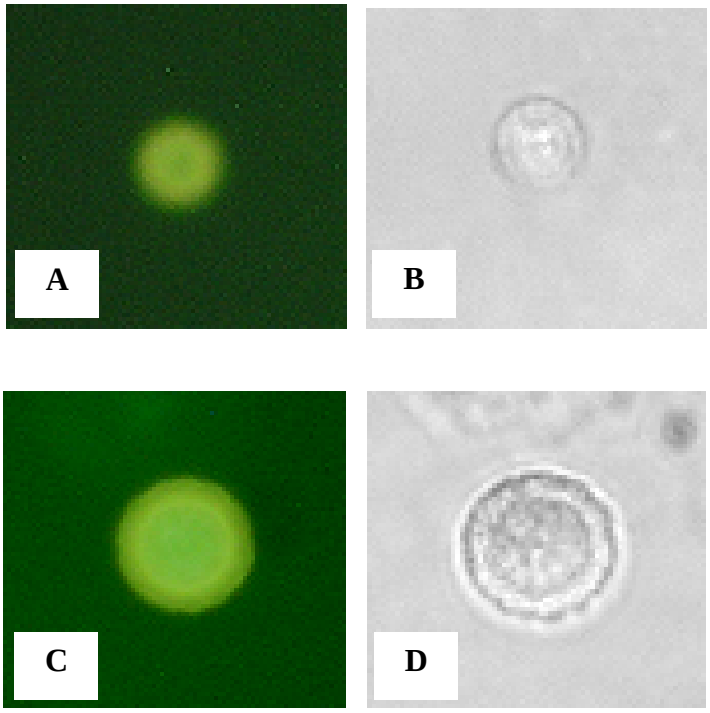


Figure 6.16 Orange-green fluorescence shown by (A) *B. mandrillaris* cysts with MAbSC62 (diluted 1:50, 200X) and (C) in the negative control with FITC-secondary antibody (400×). (B) and (D) show corresponding phase-contrast images. Similar results were also seen for MAbSC14

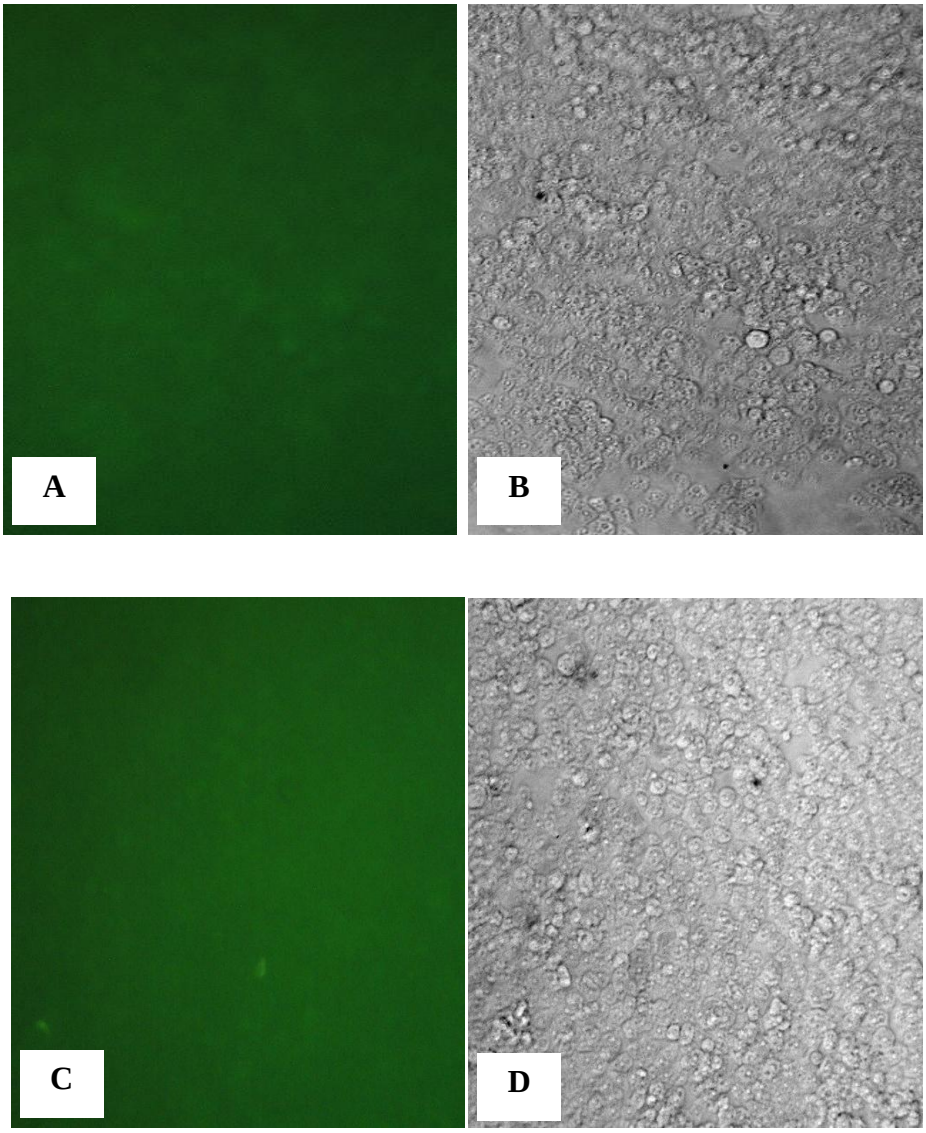


Figure 6.17 Reactivity of MAbSC62 (diluted 1:50) against *A. castellanii* trophozoites (A). A similar result was seen in well with only FITC-secondary antibody added (C). (B) and (D) show the corresponding phase-contrast images. All images are at 200×. Similar results were also seen for MAbSC14

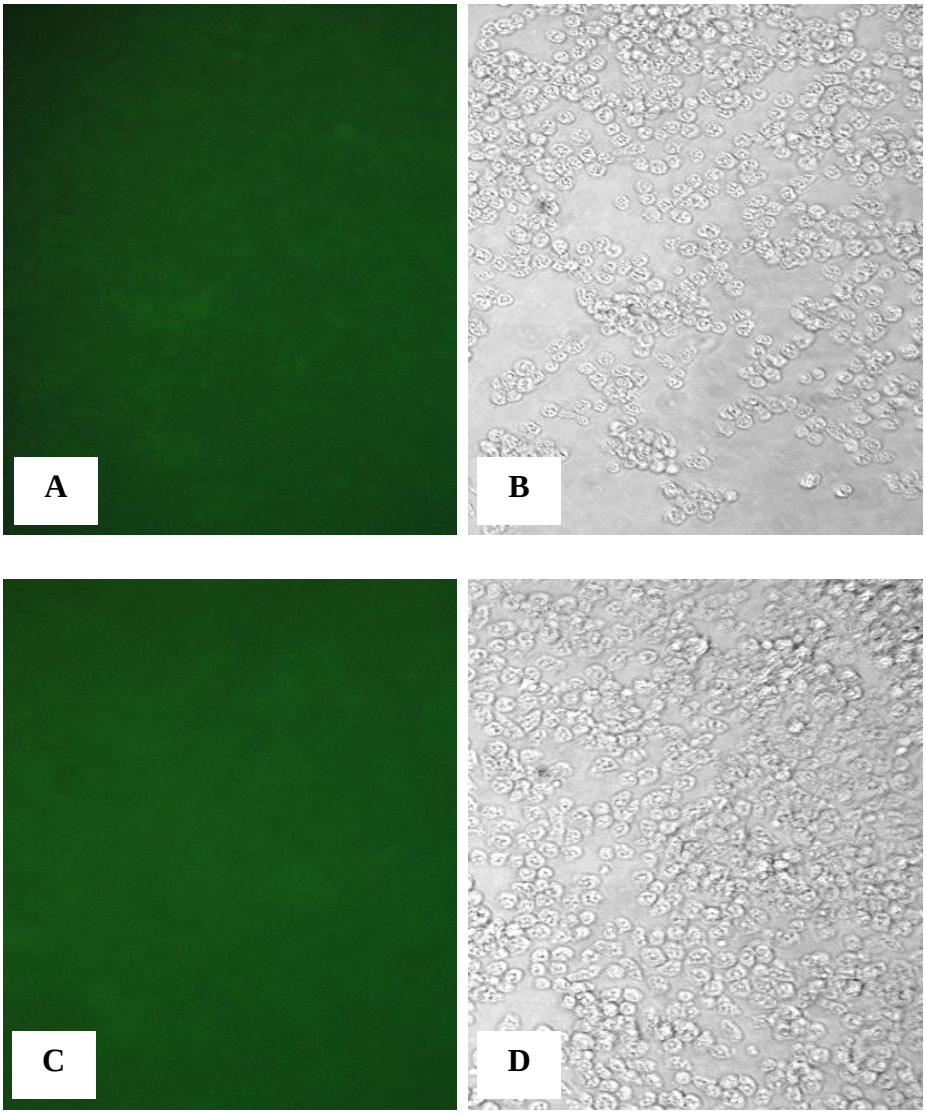


Figure 6.18 Reactivity of MAbSC62 (diluted 1:50) against *N. gruberi* trophozites (A). A similar result was seen in well with only FITC-secondary antibody added (C). (B) and (D) show the corresponding phase-contrast images. All images are at 200×. Similar results were also seen for MAbSC14

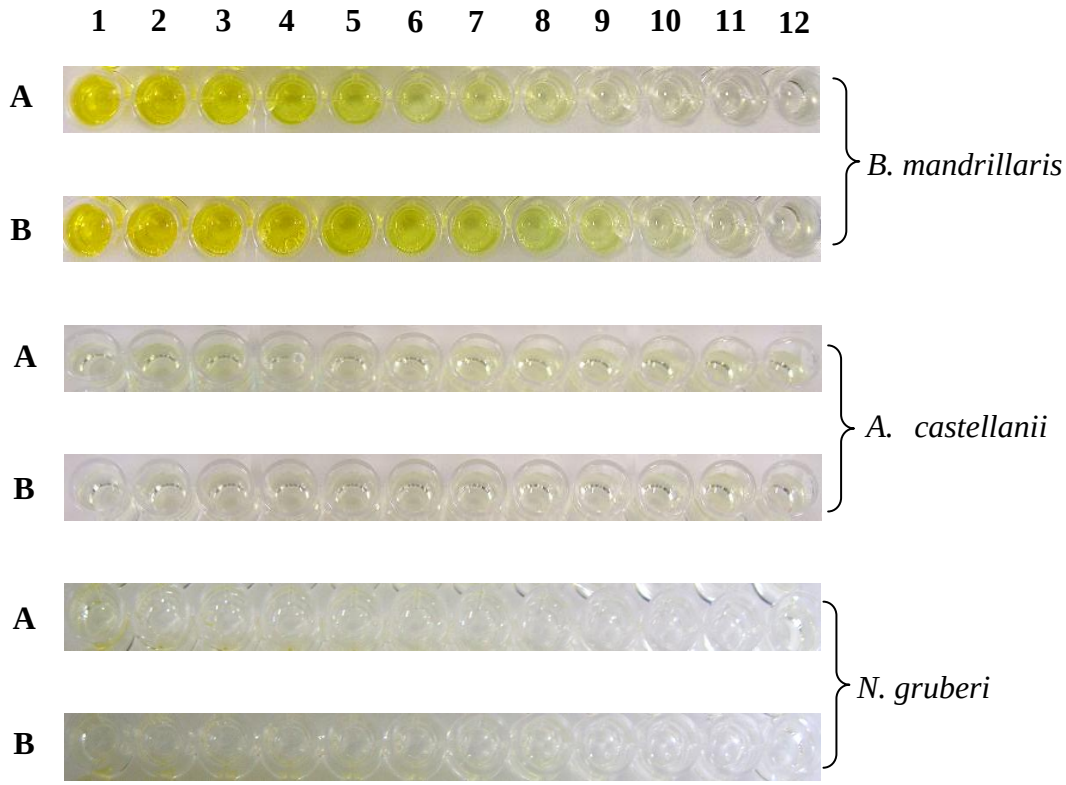


Figure 6.19 Specificity of monoclonal antibodies MAbSC62 (A) and MAbSC14 (B) against free-living amoebae by ELISA. Positive reactivity (yellow colouration) was only observed against *B. mandrillaris* trophozoites. No reactivity was shown against *A. castellanii* and *N. gruberi* trophozoites. Well 1: 1/50; Well 2: 1/100; Well 3: 1/200; Well 4: 1/400; Well 5: 1/800; Well 6: 1/1600; Well 7: 1/3200; Well 8: 1/6400; Well 9: 1/12,800; Well 10: 1/25,600; Well 11: 1/51,200 and Well 12: 1/102,400

6.2.7 Reactivity of polyclonal antibody UK4237 and monoclonal antibodies MAbSC62 and MAbSC14 with *B. mandrillaris* cysts

The reactivity of *B. mandrillaris* poly- and monoclonal antibodies produced with *B. mandrillaris* cysts were also determined by ELISA. Strong positive reactions were seen when polyclonal antibody UK4237 at dilutions 1:50 to 1:102,400 were reacted with the cysts (Figure 6.20A). Reactivity of similar dilutions of monoclonal antibodies MAbSC62 (Figure 6.20B) and MAbSC14 (Figure 6.20C) against *B. mandrillaris* cysts showed no reaction.

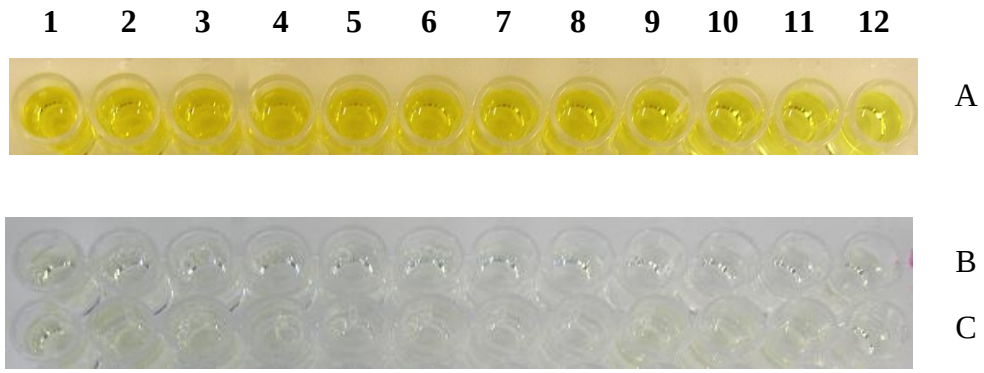


Figure 6.20 Reactivity of various dilutions of polyclonal antibody UK4237 (A) and monoclonal antibodies MAbSC62 (B) and MAbSC14 (C) with *B. mandrillaris* cysts. Well 1: 1/50; Well 2: 1/100; Well 3: 1/200; Well 4: 1/400; Well 5: 1/800; Well 6: 1/1600; Well 7: 1/3200; Well 8: 1/6400; Well 9: 1/12,800; Well 10: 1/25,600; Well 11: 1/51,200 and Well 12: 1/102,400

6.2.8 Effect of polyclonal antibody PAb UK4237 on *B. mandrillaris* trophozoites

Prior to experiment using the Dynabeads, *B. mandrillaris* trophozoites were incubated for 24 hrs in BM3 medium containing three different concentrations (5 mg/ml, 0.5 mg/ml and 0.05 mg/ml) of polyclonal antibody PAb UK4237. This was to check whether the polyclonal antibody could cause any adverse effects. Many large clumps of dead cells were seen in the flask containing the highest concentration (5 mg/ml) of PAb UK4237 (Figure 6.21A). Flasks containing 0.5 mg/ml and 0.05 mg/ml PAb UK4237 showed mainly normal branching *B. mandrillaris* trophozoites (Figure 6.21B and 1.1C) with a few small clumps of dead cells detected in the flask with 0.5 mg/ml PAb UK4237 (Figure 6.21B). Figure 6.21D shows the normal branching morphology of *B. mandrillaris* trophozoites in the control flask containing no PAb UK4237.

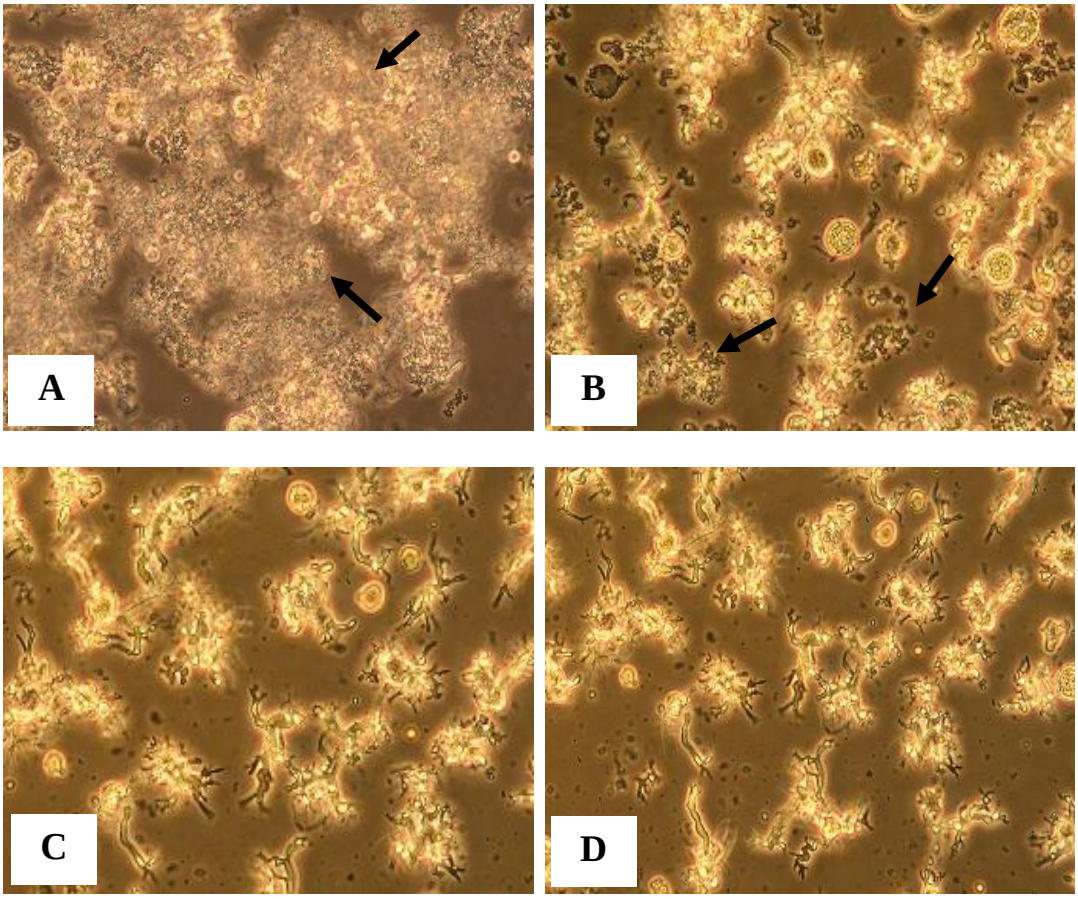


Figure 6.21 Morphology of *B. mandrillaris* trophozoites when incubated for 24 hrs with various concentrations of polyclonal antibody PAb UK4237. A: 5 mg/ml; B: 0.5 mg/ml; C: 0.05 mg/ml D: control with no PAb UK4237 added. Arrows show clumps of dead cells. All images are at 200×

6.2.9 Isolation of *B. mandrillaris* trophozoites using Dynabeads FlowComp Flexi kit and biotinylated polyclonal antibodies

Only PAb UK4237 was used for isolation of *B. mandrillaris* trophozoites using Dynabeads FlowComp Flexi kit while PAb UK4238 was kept at -80°C for further use or as a back-up. As the Dynabeads were pre-coated with streptavidin, the PAb UK4237 needed to be labelled with DSB-X biotin prior to the isolation procedure. Based on the results obtained in Section 6.2.8, a concentration of 0.15 mg/ml of biotinylated polyclonal antibody was used with the kit. Incubation of *B. mandrillaris* trophozoites

with biotinylated PAb UK4237 at a final concentration of 0.15 mg/ml in BM3 for 24 hrs did not cause any morphological changes and no clumps of dead cells were seen.

Initial experiment with Dynabeads FlowComp Flexi kit was performed according to the manufacturer's protocol except centrifugation was changed from 350 × *g* to 400 × *g*. Results showed that the end product contained mainly ruptured *B. mandrillaris* with very few viable trophozoites, which eventually died after 24 hrs of incubation in BM3 medium (Figure 6.22).

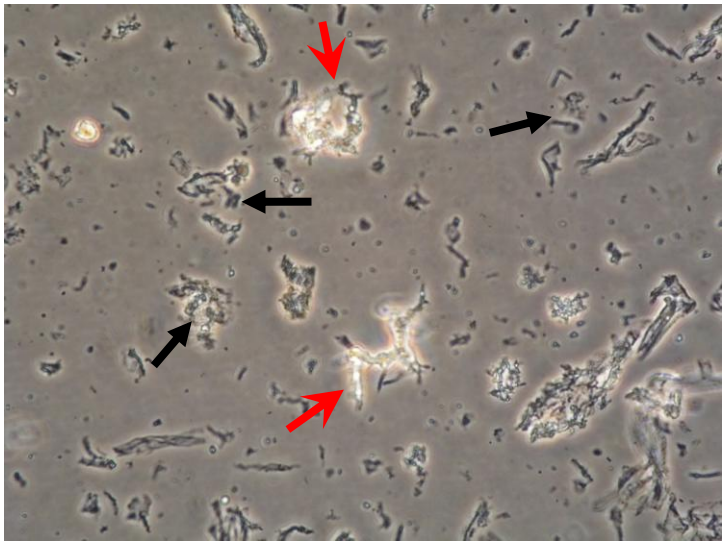


Figure 6.22 Isolation of pure *B. mandrillaris* trophozoites with Dynabeads FlowComp Flexi kit showing mostly ruptured and dead trophozoites (Black arrows). Red arrows show viable trophozoites

As the EDTA present in the isolation buffer or sodium azide present in the Dynabeads suspension was initially thought to be the cause of the dead of the trophozoites, a few changes were made. The isolation of *B. mandrillaris* trophozoites was subsequently done using a modified isolation buffer that lacked the EDTA. In addition, the Dynabeads was washed by centrifugation with the modified isolation buffer to remove the sodium azide and was resuspended in the same buffer. However, similar result was obtained, with almost all trophozoites being ruptured and dead. Therefore, to troubleshoot the cause of trophozoites death after the isolation procedure, several tests were performed (Sections 6.2.9.1 to 6.2.9.4).

6.2.9.1 Incubation of *B. mandrillaris* trophozoites in different isolation buffers

B. mandrillaris trophozoites were pre-incubated with unmodified and modified buffers to determine the best condition for the trophozoites during the isolation process. For this, trophozoites were pre-incubated for 50 min in the respective isolation buffer (Table 6.9), harvested by centrifugation and the morphology of the trophozoites was determined after incubation of the treated trophozoites in BM3 for 24 hrs. Trophozoites were considered as dead when ruptured or rounded and non-refractile cells were seen. The results are summarised in Table 6.9. The morphology of *B. mandrillaris* trophozoites was the best when pre-incubated in BM3 medium (control) where all trophozoites remained in the normal branching morphology. Trophozoites that were pre-incubated in unmodified isolation buffer (DPBS, 0.1% (w/v) BSA and 2 mM EDTA), and modified isolation buffer IB1 (DPBS and 0.1% (w/v) BSA) were mostly dead, although a few surviving trophozoites were seen. Those *B. mandrillaris* that were pre-incubated with modified isolation buffer IB2 (DPBS only) showed a moderate condition, with a mix of dead and viable trophozoites being seen.

Table 6.9. Morphology of *B. mandrillaris* trophozoites in BM3 medium following pre-incubation with unmodified and modified isolation buffers

Isolation buffer used in pre-incubation	**Morphology of *B. mandrillaris* trophozoites in BM3**
Unmodified isolation buffer containing DPBS, 0.1% (w/v) BSA and 2 mM EDTA	±
Modified isolation buffer IB1 containing DPBS and 0.1% (w/v) BSA	+
Modified isolation buffer IB2 containing DPBS only	++
BM3 (control)	+++

± = mostly dead with very few viable rounded trophozoites

\+ = mostly dead with few showing normal branching morphology trophozoites

++ = dead cells with more viable trophozoites

+++ = normal branching trophozoites

6.2.9.2 Incubation of *B. mandrillaris* trophozoites with unwashed and washed Dynabeads

Subsequently, to check whether the magnetic beads could also affect the viability of the *B. mandrillaris* trophozoites, the trophozoites were incubated for 15 min in BM3 medium containing unwashed Dynabeads (supplied by the manufacturer) or washed Dynabeads (2× centrifugation in BM3 medium). Following this, the suspension was centrifuged and pellet was incubated in fresh BM3 medium for up to 24 hrs. As the morphology of the trophozoites was hard to see because of being overcrowded with the Dynabeads, samples of the suspension were put onto glass slides for photography.

Similar normal branching morphology of *B. mandrillaris* trophozoites was observed following pre-incubation in unwashed and washed Dynabeads (Figure 6.23).

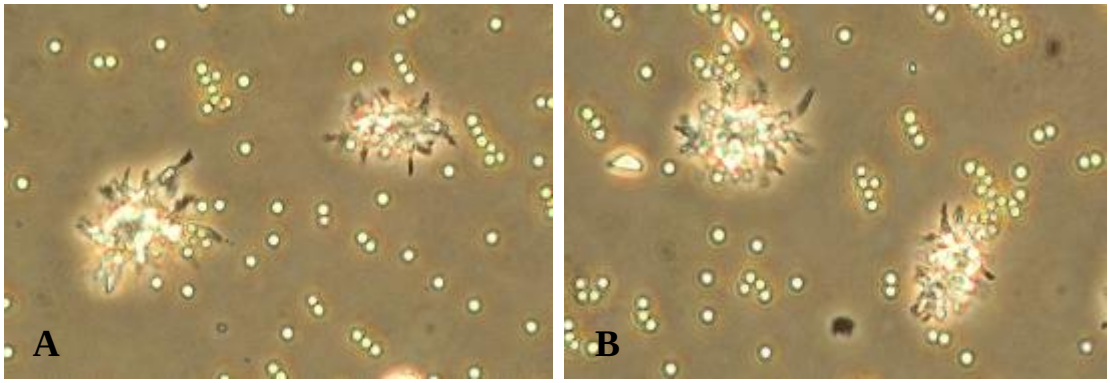


Figure 6.23 Normal branching morphology of *B. mandrillaris* trophozoites was seen after pre-incubation with unwashed (A) and washed (A) Dynabeads (400×)

6.2.9.3 Effect of release buffer on *B. mandrillaris* trophozoites

In order to test the effect of the release buffer used for separation of bound *B. mandrillaris* trophozoites from the Dynabeads, the trophozoites were incubated in the release buffer for up to 15 min and any morphological changes at time points of 2 min, 5 min, 10 and 15 min were observed (Table 6.10). At 2 min, the trophozoites were seen to be swollen with short pseudopodia were starting to become rounded. By 5 min of incubation, rounded *B. mandrillaris* trophozoites were mostly observed and a few were dead. Further incubation, for up to 15 min, revealed mainly dead trophozoites. *B. mandrillaris* trophozoites incubated in BM3 medium (control) showed normal branching morphology.

Table 6.10 Morphology of *B. mandrillaris* trophozoites after incubation in release buffer for different times

Condition of *B. mandrillaris* trophozoites	**Time point (min)**			
	2	**5**	**10**	**15**
Incubation in release buffer	++	+	±	±
Incubation in BM3 medium (control)	+++	+++	+++	+++

± = mostly dead trophozoites

\+ = rounded with few dead trophozoites

++ = swollen and started to rounded

+++ = normal branching morphology trophozoites

6.2.9.4 Isolation of *B. mandrillaris* trophozoites without the use of release buffer

Based on the results obtained in the previous sections, isolation of *B. mandrillaris* trophozoites was later performed using the BM3 medium as the isolation buffer, washed Dynabeads and without the use of release buffer. Isolated trophozoites were resuspended in BM3 medium and incubated for up to 48 hours. Normal branching trophozoites were initially observed following incubation for 10 min in the BM3. However, most of the trophozoites were dead and appeared as large aggregates following incubation for up to 48 hrs in BM3 medium.

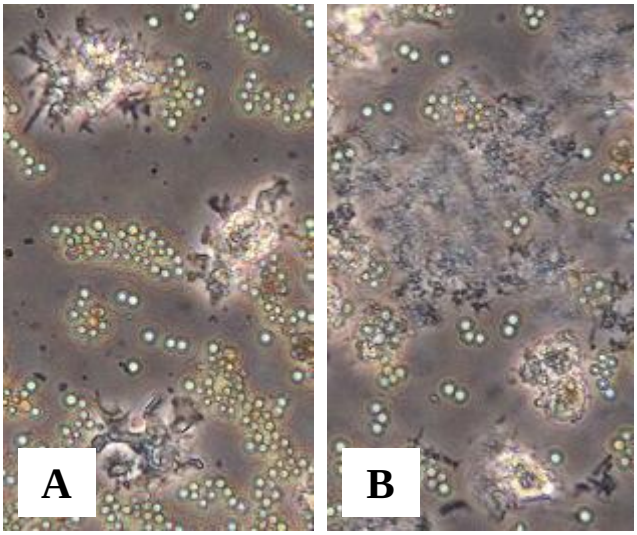


Figure 6.24 *B. mandrillaris* trophozoites isolated without the release buffer step showing the normal branching morphology following 10 min incubation in BM3 (A) and aggregates of dead amoebae seen after 48 hrs of incubation (B) (400×)

6.2.10 Isolation of *B. mandrillaris* cysts using Dynabeads FlowComp Flexi kit and biotinylated polyclonal antibodies

As *B. mandrillaris* cysts are thought to be more resistant than the trophozoites, isolation was performed using the modified isolation buffer IB1 containing DPBS and 0.1 (w/v) BSA, unwashed Dynabeads and incubation in release buffer for 2 min, instead of the recommended 10 min. The biotinylated polyclonal antibody used was PAb UK4237. The viability of the isolated cysts was determined by incubating the cysts on a monolayer of monkey kidney cells. Following incubation for up to 10 days, clearing of the monkey kidney cells was observed and most of the *B. mandrillaris* cysts had excysted to the branching trophozoites (Figure 6.25).

Subsequently, the procedure was applied for isolating *B. mandrillaris* cysts from spiked water samples. For this, water samples were filtered and the materials trapped on the membrane was collected and spiked with 10^4 of *B. mandrillaris* cysts. *B. mandrillaris*

cysts were able to be isolated but were not inoculated on monolayers of monkey kidney cells due to repeated fungal contamination. Further attempts to remove the fungal contamination using a mixture of antibacterial and antifungal from Sigma-Aldrich were unsuccessful (Figure 6.26).

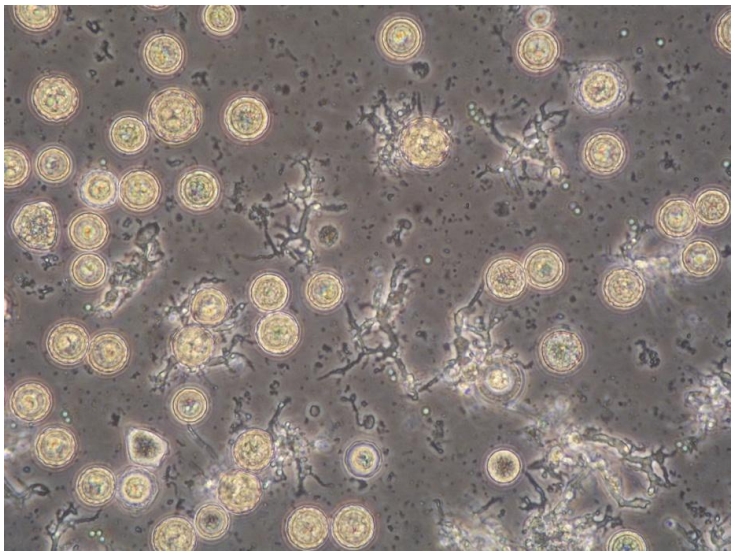


Figure 6.25 Culture of *B. mandrillaris* cysts isolated by the Dynabeads FlowComp Flexi showing clearance of monkey kidney cells and present of normal branching *B. mandrillaris* trophozoites (400×)

Figure 6.26 Fungal contamination seen in culture of *B. mandrillaris* cysts isolated from spiked water samples

6.2.11 Use of monoclonal antibodies for isolation of *B. mandrillaris* using Dynabeads FlowComp Flexi kit

Due to the inability of the *B. mandrillaris* trophozoites to survive the isolation procedure using the Dynabeads kit and polyclonal antibodies, biotinylation of monoclonal antibodies for use with the kit was not continued. Furthermore, isolation of *B. mandrillaris* cysts using the monoclonal antibodies could not be performed because they were only specific against *B. mandrillaris* trophozoites and not the cysts.

6.3 Discussion

6.3.1 Production and characterisation of poly- and monoclonal antibodies against *B. mandrillaris*

Polyclonal antibodies have been used as an important diagnostic tool for identification of infections caused by free-living amoebae, including *Balamuthia mandrillaris*, *Naegleria fowleri* and pathogenic *Acanthamoeba* species (da Rocha-Azevedo *et al.*, 2009). Monoclonal antibodies against *N. fowleri* and pathogenic *Acanthamoeba* spp. have also been produced for improving the diagnosis of infections caused by the two amoebae (Visvesvara *et al.*, 1987, Sparagano *et al.*, 1993, Turner *et al.*, 2005). However, no *B. mandrillaris* monoclonal antibodies have been developed to date. In this study, two polyclonal antibodies (UK4237 and UK4238) and two monoclonal antibodies (MAbSC62 and MAbSC14) to formalin-treated *B. mandrillaris* trophozoites were successfully produced. The production of both types of antibodies was done in collaboration with Cambridge Research Biochemicals, with all the animal work and first screening of hybridoma clones being done there, while preparation of *B. mandrillaris* immunogen, determination of polyclonal antibodies titres, second screening of hybridoma clones and specificity testing were done at the University of Leicester. In this study, it was decided to produce both *B. mandrillaris* poly- Ab and monoclonal antibodies to allow determination of the most suitable antibodies to be used for immunomagnetic separation of the amoeba using Dynabeads.

The advantages of polyclonal antibodies are they can be made within a few months, are inexpensive to produce and require less technical skill than the production of monoclonal antibodies, which can take up to a year or more and involves many stages that need technical skill and they are expensive to produce (Lipman *et al.*, 2005). It

should be noted that the main advantages of monoclonal antibodies are their high homogeneity and consistency which means all batches derived from the same source of hybridomas will generate identical monoclonal antibodies. However, this monospecificity characteristic may cause the monoclonal antibody to become too specific and minute changes in the epitope's structure may make it unrecognisable by the monoclonal antibody (Lipman *et al.*, 2005). In contrast, polyclonal antibodies are heterogeneous and prone to batch variability but because they can recognise many epitopes, a change of a single epitope may not have a significant effect on the recognition by the polyclonal antibody pool (Lipman *et al.*, 2005).

Both polyclonal antibodies (UK4237 and UK4238) developed in this study showed a strong antibody-antigen reaction against *B. mandrillaris* antigen, even at the highest dilution used (1:102,400). However, a specificity test performed with polyclonal antibody UK4237 showed that a positive reaction was also seen with *A. castellanii* and *N. gruberi* trophozoites presumably due to shared epitopes. It was noticed that the specificity results could vary depending on the sensitivity of assays used. For example, the results of immunoperoxidase assays, as judged by the intensity of blue staining produced, showed that PAb UK4237, at a dilution of 1:2500, showed cross-reactivity to *A. castellanii* but not to *N. gruberi* trophozoites. However, reactivity against *N. gruberi* was detected by ELISA using more diluted PAb UK4237 (1:3200), suggesting this assay was more sensitive than the immunoperoxidase staining method. Besides being more sensitive, ELISA has the advantage in that results of the assay can either be interpreted visually, based on the intensity of the colour produced as compared to the controls, or quantitatively by measuring the absorbance. This advantage could be of benefit to developing countries in which not all laboratories are equipped with a spectrophotometer. Also, if the machine breakdown occurs one can still estimate the

antigen-antibody reaction based on visual inspection of the intensity of the colour produced.

An advantage of using an immunofluorescence assay was the ability to show whether the antibodies were reactive to the cell membrane or whole *B. mandrillaris* trophozoites. Specificity testing performed with this assay showed that cross-reactivity with *A. castellanii* and *N. gruberi* trophozoites could be eliminated when PAb UK4237 was diluted to ≥ 1:3200. However, it was noticed that this assay was not suitable for use against *B. mandrillaris* cysts due to autofluorescence of the cysts. Problems with autofluorescence have been reported with cysts of an intestinal protozoon, *Jodamoeba bütschlii* during a study with monoclonal antibodies developed against *Entamoeba histolytica* cysts (Walderich *et al.*, 1998). A disadvantage of immunofluorescence assays is interpretation of results, which are judged by the degree of fluorescent seen and this can be subjective and vary from individual to individual. Furthermore the assay requires the use of an expensive immunofluorescence microscope and the bright green fluorescent produced can be stressful to the eyes.

As shown by the ELISA results, PAb UK4237 showed reactivity against *B. mandrillaris* trophozoites and also to the cysts. This phenomenon is not surprising because it has been reported that *Acanthamoeba* cysts and trophozoites exhibit common antigens but the expression of the antigen is reduced in the cysts because most of the protein are lost towards the end of the encystment process (McClellan *et al.*, 2002). In the study, it was shown that serum from mice immunised with whole *Acanthamoeba* trophozoites showed similar reactivity against both the trophozoites and cysts but serum from mice immunised with the same preparation of cysts showed less reactivity against *Acanthamoeba* trophozoites than the cysts, as determined by ELISA, thus suggesting the lack of certain antigens on the cysts (McClellan *et al.*, 2002). In another study,

Turner *et al*. (2005) showed that two monoclonal antibodies derived from heat-treated *Acanthamoeba* trophozoites and membrane preparation of the trophozoites were able to show reactivity against both the trophozoite and cyst stages by flow cytometry (Turner *et al.*, 2005).

In the production of *B. mandrillaris* monoclonal antibodies, it was shown that low antibodies titres were obtained from test-bleed 1 and test-bleed 2 sera as determined by ELISA with wells coated with whole-cells *B. mandrillaris*, performed at Cambridge Research Biochemicals (CRB). As it was initially thought that the low titres were due to problems with the type of antigen used to coat the ELISA plates, it was later decided in consultation with CRB that screening of test-bleed 3 sera should be performed with plates coated with either whole-cells or lysed *B. mandrillaris* for comparison purposes. As shown by the results in Table 6.5, the antibodies titres obtained by CRB were higher with plates coated with whole-cells rather than the lysed *B. mandrillaris*. Therefore, it was concluded that the low titres were not due to problems with the antigen used for coating the ELISA plates but possibly were due to mice that had not fully developed *B. mandrillaris* antibodies at the time test-bleeds 1 and 2 were taken. In contrast to the data from CRB, a second ELISA screening performed at the University of Leicester showed similar ELISA results when serum from test-bleed 3 of mouse UK4245 were tested with plates coated either with whole-cells or lysed *B. mandrillaris*. The differences between the results obtained by the CRB and University of Leicester could be due to the different method used in interpreting the ELISA results. The results by the CRB were interpreted based on absorbance readings while those done at the University of Leicester were based on visual inspection of the intensity of the colour reaction produced. To avoid further conflict as to which type of antigen should be used for coating the ELISA plates, it was decided that all screening of hybridoma clones done at

CRB were to be done with whole-cells of *B. mandrillaris*, while those performed at University of Leicester were done against lysed *B. mandrillaris*. The results from both ELISA screenings were then compared before a decision was made for subsequent phases of monoclonal antibodies production. As shown in the results, those hybridoma clones that showed high antibody titre by CRB, also were shown to have similar results in ELISA screening done at the University.

Monoclonal antibodies (MAbSC62 and MAbSC14) developed here were characterised as isotype IgG1. IgG isotypes play important roles in complement fixation, opsonisation and fixation to macrophages (Buckley, 1998). The advantages of IgG1 isotype are they have the highest affinity for C1q compared to the other IgG isotypes, higher affinity to Fc receptors on phagocytic cells than IgG2 and IgG4, less susceptibility to proteolytic digestion and long serum half-life (23 days) (Buckley, 1998). Due to these advantages, IgG1 has been preferred for development of therapeutic agents (Salfeld, 2007).

Based on immunofuorescence and ELISA results shown in Section 6.2.6, both monoclonal antibodies were confirmed to be specific against *B. mandrillaris* trophozoites and no cross-reactivity was seen against *A. castellanii* and *N. gruberi*. This suggests that both monoclonal antibodies may have diagnostic value for differentiating infection caused by *B. mandrillaris* from those by other free-living amoebae. To date, confirmation of BAE cases are mainly by immunofluorescence assay of brain sections with *B. mandrillaris* polyclonal antibodies (Deetz *et al.*, 2003, Prasad *et al.*, 2008). It would therefore be interesting if the monoclonal antibodies derived here could further be tested against brain sections of patients suspected to have BAE to test their usefulness.

In this study, both monoclonal antibodies produced showed better specificity against *B. mandrillaris* trophozoites than the polyclonal antibodies. As mentioned on page 250, variation in the results from testing the specificity of polyclonal antibodies (PAbs) could be due to dilution of PAb and type of assays used. For example, PAb UK4237 at a dilution of 1:2500 showed cross-reactivity to *A. castellanii* but not to *N. gruberi* trophozoites in immunoperoxidase assays, but in ELISAs, PAb UK4237 at a lower dilution (1:3200), showed reactivity against both *A. castellanii* and *N. gruberi* trophozoites. In contrast, PAb UK4237 at a dilution of 1:3200 showed no reactivity with both of the amoebae in immunofluorescence assays. The findings from these specificity testings suggest that the dilution of polyclonal antibodies used and utilisation of more than one type of assay is important when determining the specificity of antibodies and to eliminate false-positive results due to cross-reactivity against other free-living amoebae. This is important, especially if the polyclonal antibodies would be used for diagnosis of encephalitis that can be caused by any of the three different genera of free-living amoebae.

6.3.1.1 Problems encountered

Preparation of *B. mandrillaris* immunogen was a challenging process because, unlike *Acanthamoeba* which can easily be grown in a large-scale in an axenic medium, the *B. mandrillaris* used were not able to be adapted for growth in BM3 axenic medium during the preparation of the immunogen Therefore, the *B. mandrillaris* needed to be cultured on mammalian cells as the food source. This could take up to five days for the amoeba to feed on the cells prior to an increase in *B. mandrillaris* numbers. As shown in Chapter 3, the monkey kidney cells (MA104) had been chosen to grow the amoeba, however it should be noted that *B. mandrillaris* has a long doubling-time and does not produce a confluent layer on the surface of the flask, in contrast to *Acanthamoeba*. As a

consequence, although high numbers of *B. mandrillaris* was obtained, they never were as high as those that could be produced with *Acanthamoeba* (personal observation) and multiple culturing needed to be done to obtain sufficient immunogen for production of antibodies. Furthermore, the *B. mandrillaris* needs to be grown in a medium that is free from antibiotics and antifungals (personal communication with Prof Naveed Khan, University of Nottingham) because they can inhibit the growth of the amoeba. This made the cultures proned to contamination. Although caution was taken by performing any manipulations of *B. mandrillaris* in a class II safety cabinet, contamination still could occur. Hence, more time had to be spent on the culturing and preparation of *B. mandrillaris* immunogen then what scheduled at the start of the project.

In this study, the specificity assays were performed with a 96-well plate instead of Teflon glass slides, which originally was the desired option. This was because, unlike *Acanthamoeba* and *Naegleria* which could adhere easily to the glass slide, *B. mandrillaris* trophozoites showed poor adherence and attempts to prolong the time for adherence resulted in changes in the morphology of the amoeba. Besides solving the problem of adherence, assays performed using the 96-well plate offer an advantage that cross-contamination of the contents between wells could be avoided, in contrast to Teflon slides where contamination is commonly encountered if the areas between wells are not dried adequately.

As only one strain of *B. mandrillaris* (ATCC 50209) was available at the American Type Culture Collection, specificity testing was done only against this strain. Attempts to obtain other clinical and environmental *B. mandrillaris* strains from other research group in Peru and Centres for Diseases Control and Prevention (CDC) in USA were unsuccessful.

6.3.2 Application of anti-*B. mandrillaris* antibodies in immunomagnetic separation

In this study, polyclonal antibody UK4237 was used in combination with a Dynabeads FlowComp Flexi kit to determine their suitability in immunomagnetic separation of *B. mandrillaris* trophozoites and cysts. The general principles for immunomagnetic separation involve incubation of a mixture of cells with specific antibodies bound to magnetic beads, separation of target cells-magnetic beads complexes using a magnet separator and finally detachment of target cells from the magnetic beads (Safarik *et al.*, 1999).

Cell isolation using magnetic particles gained attention in the 1970s because the method is easy to perform and fast. Magnetic particles are available from many manufacturers, in various forms and sizes from <1 to 5 µm (Safarik *et al.*, 1999). One of the most popular magnetic particles used in cell isolation are Dynabeads (Dynal, Oslo, Norway). Dynabeads are monosized polystyrene beads invented by Professor John Ugelstad in 1977. The beads are magnetisable and are superparamagnetic meaning that they are only magnetic in a magnetic field (Neurauter *et al.*, 2007). Generally cell isolation can be performed by positive isolation, negative isolation or combination of both. Positive isolation involves isolation of the target cell type from crude samples using Dynabeads coupled with a ligand with an affinity for the target. In negative isolation, all unwanted cells are first removed by Dynabeads which results in the target cells being left. Both types of isolation can be performed either by a direct or indirect method. The direct method involves sensitisation of Dynabeads with a specific ligand prior to the isolation procedure. Whereas, indirect method requires target cells to be first sensitised with a suitable primary affinity ligand prior to isolation using Dynabeads coated with a secondary ligand or antibody (Neurauter *et al.*, 2007).

Positive isolation of target cells has been reported to be challenging because the target cell might be affected or altered during the isolation process (Neurauter *et al.,* 2007). In this study, it was shown that the immunomagnetic separation assay has limited use in the isolation of *B. mandrillaris* trophozoites. This could be because the trophozoites are very sensitive to changes in the environment conditions, in contrast to the cysts (Visvesvara *et al.*, 2007, Siddiqui *et al.*, 2008b). As shown by the results in Section 6.2.9, most of the trophozoites were dead following incubation in different isolation buffers, except in the axenic BM3 medium used to grow the amoeba. In addition, *B. mandrillaris* trophozoites also showed sensitivity to the release buffer used to separate the captured amoebae from the Dynabeads, as evidenced by the morphological changes of the trophozoites that were observed by 2 min of incubation and further incubation resulted in death of amoeba.

The positive isolation of *B. mandrillaris* trophozoites was eventually performed in BM3 medium, with the release buffer step being omitted to avoid conditions unfavourable to the trophozoites. As shown in the results, trophozoites with the normal branching morphology were initially observed following the isolation process, however further incubation in the BM3 medium for up to 48 hours resulted in death of the trophozoites. As the immunomagnetic separation was a multi-step process, it was unknown which part of the process was responsible for the death of the *B. mandrillaris* trophozoites. One possibility is that the death could be due to iron oxide exposure from the magnetic beads during incubation in BM3 medium. Studies have shown that temporary exposure of cells to moderate concentrations of iron oxide could affect cell function, phenotype and viability (Berry *et al.*, 2003, Pisanic *et al.*, 2007). However this was unlikely to happen here because each of the Dynabeads is coated with a polymer shell to prevent iron oxide leakage. As the antibodies used in this study remained attached to the

trophozoites following the isolation procedure, the death could also be due to signalling generated by the antibodies through the cell surface molecules. It has been reported that antibody-antigen binding might lead to clustering of receptors, triggering of positive or negative signalling pathways or even blocking of receptor function (Neurauter *et al.*, 2007).

In contrast, *B. mandrillaris* cysts were shown to be able to survive the harsh conditions during the immunomagnetic separation process, as evidenced by excystment of *B. mandrillaris* cysts following incubation on MA104 monolayer in control experiments. This could be due to the morphology of *B. mandrillaris* cysts which has triple-layered wall, termed as ectocyst, mesocyst and endocyst (Visvesvara *et al.*, 2007), that protected them from unfavourable conditions. It was noticed that when immunomagnetic separation was performed with *B. mandrillaris* cysts-spiked water samples, the isolated cysts often became contaminated with fungi which hindered the confirmation of cysts viability using MA104 monolayer. In this study, fungal contamination of cultures of isolated *B. mandrillaris* cysts could not be eliminated, even after treatment with an antifungal mixture from Sigma-Aldrich. Therefore, subsequent steps to determine the viability of *B. mandrillaris* cysts by inoculation on mammalian cells could not be continued. If there had been sufficient time, different concentrations of the antifungal mixture would have been tested for elimination of the fungal contamination. It is concluded that the technique has potential value to be applied for the isolation of *B. mandrillaris* cysts, provided that the fungal contamination can be overcome.

7 *In vitro* drug sensitivity study against *B. mandrillaris* trophozoites and cysts

7.1 Introduction

The lack of suitable treatment for *B. mandrillaris* diseases has resulted in few survivors (Schuster *et al.*, 2004d, Martinez *et al.*, 2010). Frequently, the treatment involves a combination of drugs including flucytosine, fluconazole, pentamidine isethionate, sulfadiazine, macrolide antibiotics (azithromycin or clarithromycin) and phenothiazines (thioridazine or trifluoperazine) (Deetz *et al.*, 2003, Jung *et al.*, 2004, Cary *et al.*, 2010). Nevertheless, the same drug regimen might not be effective against all balamuthiasis patients and some drugs can cause side-effects (Seas *et al.*, 2006, Deetz *et al.*, 2003, Cary *et al.*, 2010).

The selection of drugs of choice is normally made based on drug efficacy of individual drug when tested against *B. mandrillaris* by *in vitro* assays (Schuster *et al.*, 1996, Schuster *et al.*, 2008a). As discussed in Section 1.1.5.1, the drug assays which are normally performed using *B. mandrillaris* trophozoites can be done in a cell-free culture medium or on mammalian cell cultures (Schuster *et al.*, 1996, Schuster *et al.*, 2006a). The amoebastatic or amoebacidal effect of a drug is then be confirmed by transferring the amoeba onto a fresh mammalian monolayer or incubation in a drug-free culture medium to determine the viability of the drug-treated amoeba (Schuster *et al.*, 1996, Schuster *et al.*, 2006a). In this chapter, improved *in vitro* drug assays were developed using BM3 axenic medium and tested for rapid screening of drug activity against *B. mandrillaris* trophozoites and cysts.

7.2 Results

7.2.1 Large-scale production of *B. mandrillaris* cysts

In order to determine the best way for producing *B. mandrillaris* cysts in large-scale for use in a drug sensitivity study, several methods were tested. Natural encystment from incubation of *B. mandrillaris* trophozoites on MA104 monolayers for 10 days yielded 40-50% mature cysts. Attempts to produce *B. mandrillaris* cysts on a large-scale using *Acanthamoeba* encystment media, including Neff's, NMT and epinephrine (5 mM), resulted in immature cysts with lack of cell walls. *B. mandrillaris* encystment using the recommended method (Siddiqui *et al.*, 2010) by incubating *B. mandrillaris* trophozoites in RPMI plus L-galactose (100 mM) yielded low numbers of mature cysts (<15%). Therefore, large-scale production of *B. mandrillaris* cysts was performed by the natural encystment method.

7.2.2 Toxicity of drug solvents against *B. mandrillaris*

The toxicity of the solvents used in preparation of drug solutions was evaluated by incubating *B. mandrillaris* trophozoites or cysts in 0.005-5% (v/v) methanol, 0.01-10% (v/v) ethanol and 0.05-50% (v/v) DMSO for 48 hours. Subsequently, the solvents were replaced with BM3 medium and *B. mandrillaris* incubated at 37°C. The confirmation of viability of the trophozoites or cysts was checked using inverted microscopy to determine the presence of normal branching trophozoites. Toxicity was not seen when the highest concentrations of 5% (v/v) methanol and 10% (v/v) ethanol used was incubated with *B. mandrillaris* trophozoites and cysts. DMSO showed toxicity at concentrations of 3-50% (v/v) and 6.3-50% (v/v) for *B. mandrillaris* trophozoites and cysts respectively. Toxicity was not seen when *B. mandrillaris* trophozoites and cysts

were incubated with DMSO at concentrations of ≤ 1.6% (v/v) and ≤ 3% (v/v) respectively.

7.2.3 Optimisation of drug assay with *B. mandrillaris* cysts

As *B. mandrillaris* has been shown to feed on *Acanthamoeba* trophozoites (Matin *et al.*, 2006a), it was decided to use the amoeba for triggering *B. mandrillaris* excystment and growth following drug exposure. In order to determine the suitable numbers of *Acanthamoeba* trophozoites to be added into each 96-well plate, a control experiment was performed using drug-free *B. mandrillaris* cysts. A range from 15-40 *Acanthamoeba* trophozoites was added into each well containing 1×10^4 *B. mandrillaris* cysts in BM3 medium. The 96-well plate was then incubated for 12 days and *B. mandrillaris* excystment and growth were checked using inverted microscopy. As shown in Table 7.1, *B. mandrillaris* excystment and growth was the best in wells with 15-20 *Acanthamoeba* trophozoites added. Greater numbers of *Acanthamoeba* trophozoites resulted in overcrowding of *Acanthamoeba* in wells and was inhibitory to *B. mandrillaris* excystment and growth. In control well containing only BM3 medium without *Acanthamoeba* trophozoites, poor excystment and *B. mandrillaris* growth were seen.

A separate control experiment was also performed to determine the suitable time for *Acanthamoeba* trophozoites to be added into each 96-well plate containing drug-free *B. mandrillaris* cysts. Based on the previous results, 15-20 *Acanthamoeba* trophozoites was added into each of the 96 wells. Initially 1×10^4/well *B. mandrillaris* cysts were added to three 96-well plates in BM3 medium and *Acanthamoeba* trophozoites were added into each of the wells on day-1, day-3 or day-5. Plates were then re-incubated for up to 12 days and *B. mandrillaris* excystment and growth were checked by microscopy. Table 7.2 shows that the addition of *Acanthamoeba* trophozoites on day-5 resulted in

the best *B. mandrillaris* excystment and growth. In contrast, the addition of *Acanthamoeba* trophozoites on day-1 and day-3 resulted in overgrowth of *Acanthamoeba* trophozoites and lower *B. mandrillaris* excystment and growth. Therefore, for drug assays with *B. mandrillaris* cysts, 15-20 of *Acanthamoeba* trophozoites were added into each 96 wells on day-5 to provide the best *B. mandrillaris* excystment and growth.

Table 7.1 Effect of *Acanthamoeba* trophozoites numbers on *B. mandrillaris* excystment and growth

Numbers of *Acanthamoeba* added into each well (trophozoites)	***B. mandrillaris* excystment and growth**
15-20	+++
25-30	++^
35-40	+^
Control (BM3 only)	+

+ = poor; ++ = moderate; +++ = best

^ = overcrowding of *Acanthamoeba* trophozoites in wells

Table 7.2 Effect of time of addition of *Acanthamoeba* trophozoites on *B. mandrillaris* excystment and growth

Time of *Acanthamoeba* trophozoites addition (day)	***B. mandrillaris* excystment and growth**
Day-1	+^
Day-3	++^
Day-5	+++

+ = poor; ++ = moderate; +++ = best

^ = overcrowding of *Acanthamoeba* trophozoites in wells

7.2.4 Drug activity against *Balamuthia mandrillaris*

Two methods were compared for determining the minimum trophozoite amoebacidal concentration (MTAC) values for *B. mandrillaris* trophozoites: 1) presence of normal branching *B. mandrillaris* trophozoites following incubation of drug-treated trophozoites in drug-free BM3 medium for 7 days; 2) presence of *B. mandrillaris* trophozoites and clearance of MA104 monolayer following 7 days of incubation with drug-treated trophozoites. Table 7.3 shows the MTAC values for eleven drugs against *B. mandrillaris* trophozoites and toxicity of the drugs against monkey kidney cells (MA104). Pentamidine isethionate has been demonstrated to be the most effective *in vitro* drug against *Balamuthia* trophozoites (Schuster *et al.*, 1996). In this study, the drug was used as a baseline in comparing the activity of other drugs. The MTAC of pentamidine isethionate was 500 µM. Diminazene aceturate appeared to be the most effective drugs against *Balamuthia* trophozoites with a MTAC of 7.8 µM. As shown in Figure 7.1b, the trophozoites appeared as either lysed or degenerated following 48 hours of exposure to diminazene aceturate. The normal branching morphology of *B. mandrillaris* trophozoites in the control well is shown in Figure 7.1a. Amphotericin B did not show any amoebacidal activity at the highest concentration (135.3 µM) tested. Paromomycin sulfate, ciclopirox olamine, miltefosine and protriptyline hydrochloride yielded marginal activity against the trophozoites, with MTACs of 250 µM. While, poor to no amoebacidal activities (500 µM- >500 µM) were seen for telithromycin, sulconazole nitrate and spiramycin. Natamycin showed a MTAC of 31.3 µM, however the percentage of DMSO used at this drug concentration showed toxicity against *B. mandrillaris* trophozoites. Similar MTACs results were obtained following incubation of drug treated trophozoites onto MA104 monolayer (Table 7.3).

As shown in Table 7.3, diminazene aceturate, paromomycin sulphate, spiramycin and telithromycin showed limited toxicity (250 µM - >500 µM) to MA104 cells. All other drugs showed toxicity to MA104 cells between 31.3 µM – 125 µM. The apparent toxicity of natamycin to MA104 cells at 62.5 µM was suspected to be due to DMSO and not the drug.

Table 7.4 shows the minimum cysticidal concentration (MCC) values for *B. mandrillaris* cysts when exposed to the eleven drugs. Two methods for determining the viability of the *B. mandrillaris* cysts also were compared: 1) presence of *B. mandrillaris* trophozoites due to excystment of drug-treated cysts in drug-free BM3 medium that were added with *Acanthamoeba* trophozoites; 2) presence of *B. mandrillaris* trophozoites following clearance of MA104 monolayer when incubated with drug-treated cysts. Only diminazene aceturate showed efficacy with a MCC of 62.5 µM and 31.3 for method 1 and method 2 respectively. *B. mandrillaris* showed a poorly defined cyst wall and loss of intracellular contents following 48 hours of exposure to diminazene aceturate is shown in Figure 7.1d. The morphology of the control *B. mandrillaris* cysts in drug-free BM3 medium is shown in Figure 7.1c. Both viability methods showed similar MCC values for amphotericin B (>135 µM), miltefosine (>500 µM) and spiramycin (>500 µM). All other drugs (except Natamycin) showed a MCC between 500 µM - >500 µM, as determined by method 1 and 250 µM by method 2. Natamycin showed MCC of 62.5 µM and 31.3 µM by method 1 and method 2 respectively but the percentage of DMSO used for the natamycin at 62.5 µM was tested to be toxic to *B. mandrillaris* cysts.

Table 7.3 Efficacy of drugs against *Balamuthia mandrillaris* trophozoites and their toxicity against the mammalian MA104 cell line

Drug	***MTAC (µM)**	**^MTAC (µM)**	**[#]MA104 Toxicity (µM)**
Amphotericin B soultion	>135	>135	68
Diminazene aceturate (Berenil®)	7.8	7.8	250
Ciclopirox olamine	250	250	125
Miltefosine	250	250	125
Natamycin (Pimaricin)	31.3**	31.3**	62.5**
Paromomycin sulfate	250	250	>500
Pentamidine isethionate	500	500	125
Protriptyline hydrochloride	250	250	31.3
Spiramycin	>500	>500	500
Sulconazole nitrate	500	500	125
Telithromycin	500	500	500

***MTAC** = Minimum trophozoite amoebacidal concentration following 7 days incubation in drug-free BM3

^**MTAC** = Minimum trophozoite amoebacidal concentration following 7 days incubation on MA104 monolayers

[#]**MA104 Toxicity** = Concentration at which disintegration of monolayer and/or rounding of all cells occurs

**** =** DMSO was suspected as causing toxicity to the trophozoites and monolayer rather than the drug

Table 7.4 Efficacy of drugs activity against *Balamuthia mandrillaris* cysts

Drug	***MCC (µM)**	**^MCC (µM)**
Amphotericin B soultion	>135	>135
Diminazene aceturate (Berenil®)	62.5	31.3
Ciclopirox olamine	>500	250
Miltefosine	>500	>500
Natamycin (Pimaricin)	62.5****	31.3
Paromomycin sulfate	500	250
Pentamidine isethionate	>500	250
Protriptyline hydrochloride	500	250
Spiramycin	>500	>500
Sulconazole nitrate	500	250
Telithromycin	>500	250

*MCC = Minimum cysticidal concentration following 12 days incubation in drug-free BM3 medium containing *Acanthamoeba* trophozoites

^MCC = Minimum cysticidal concentration following 12 days incubation on MA104 monolayers

**** =** DMSO was suspected as causing toxicity to the cysts and not the drug

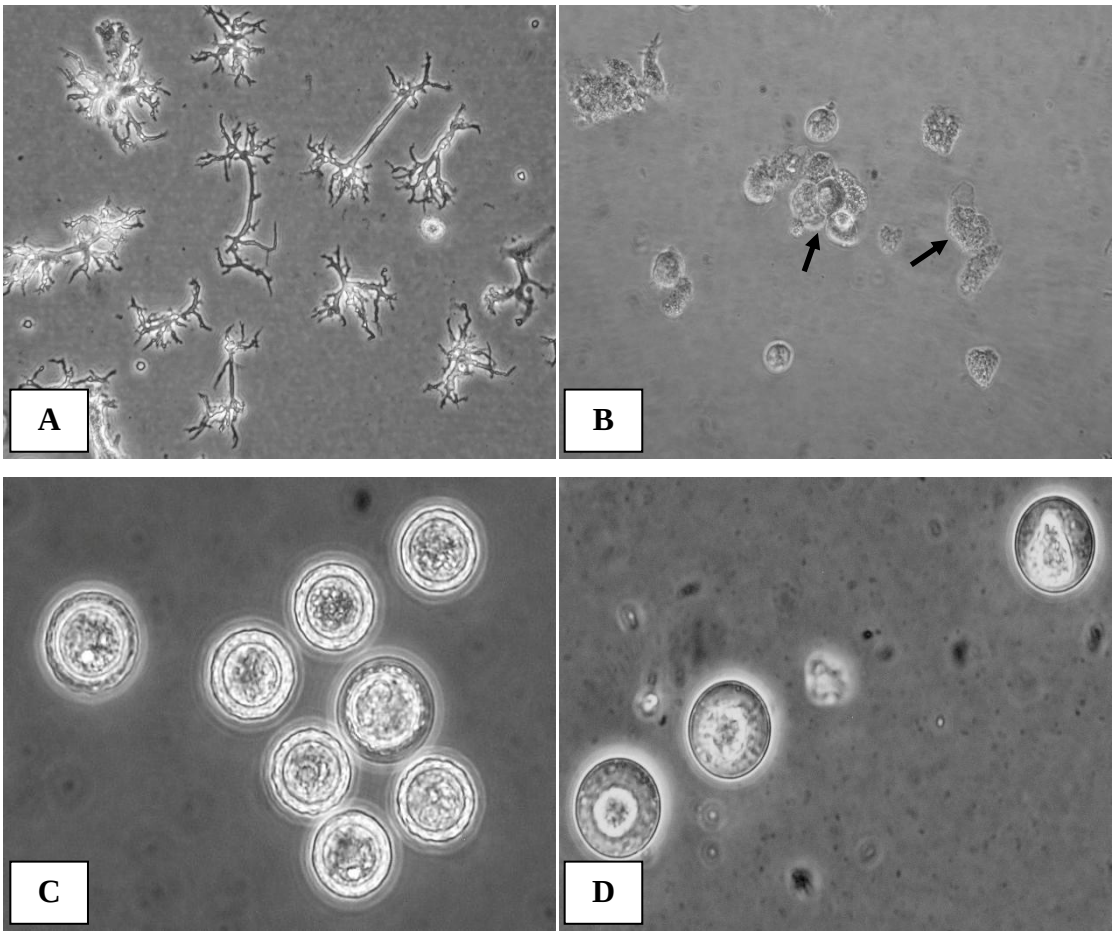


Figure 7.1 Morphology of *Balamuthia mandrillaris*. a) trophozoites in BM3 medium, b) trophozoites following 48 hr exposure to 7.8 µM diminazene aceturate (trophozoites appeared either lysed or degenerated as shown by arrows), c) cysts in BM3 medium, and d) cysts following 48 hr exposure to 62.5 µM diminazene aceturate showing a poorly defined cyst wall and loss of intracellular contents (400×)

7.3 Discussion

Previous methods of growth of *B. mandrillaris* relied on culture on mammalian cells (Schuster, 2002, Visvesvara *et al.*, 1993). However, Schuster *et al*. (1996) have described an axenic medium for growing the amoeba (Schuster *et al.*, 1996). Therefore, an improved drug sensitivity assay was developed that used axenic culture. The advantage of the drug assays developed here was that assessment of sensitivity could be performed without the use of mammalian cells. Hence the assays are less laborious and incubation of the assays can be performed in a normal incubator rather than a CO_2 incubator which requires constant maintenance and renewal of the CO_2. Here, drug efficacy against *B. mandrillaris* was based on the presence or absence of trophozoites with the normal branching morphology following drugs exposure. This has the advantage over some drugs assays in which the efficacy of drugs is assessed by the percentage of amoeba growth inhibition. Such assays require counting of amoeba to be performed at different time points (Schuster *et al.*, 1996). In addition, as the assay was performed with a 96-well plate instead of using flasks, many drugs concentrations could be tested simultaneously and more rapidly.

As shown in Table 7.3, amphotericin B and macrolide antibiotics, including spiramycin and telithromycin were not effective against *B. mandrillaris* trophozoites. These findings are in accordance with previous reports which showed that amphotericin B and other macrolides, such as azithromycin and clarithromycin, were only amoebastatic and not amoebacidal to *B. mandrillaris* trophozoites (Schuster *et al.*, 1996, Visvesvara *et al.*, 2007). In bacteria, macrolides inhibit protein synthesis by targeting the 23S ribosomal RNA molecule of the large ribosomal subunit (50S) (Mazzei *et al.*, 1993). The mechanism of actions can be through blocking the translocation of peptidyl-tRNA, inhibiting the peptidyl transfer reaction or stimulating early dissociation of peptidyl-

tRNA from the ribosomes (Mazzei *et al.*, 1993). As prokaryotes and eukaryotes have different size and structure of their ribosomes (Doudna *et al.*, 2002), this could be one of the reasons for the poor efficacy of the macrolides against the *B. mandrillaris*. The prokaryotic ribosome is 70S in size with a 50S large subunit and a 30S small subunit. In contrast, the size of eukaryotic ribosome is 80S with 60S large subunit and 40S small subunit (Doudna *et al.*, 2002, Mazzei *et al.*, 1993). It has been reported that the large subunit of the eukaryotes (e.g. *Saccharomyces cerevisiae*) contains hundreds nucleotides longer and 12 proteins more than those of prokaryotes (e.g. *Escherichia coli*) (Doudna *et al.*, 2002). Amphotericin B is the most potent drug used in the treatment of primary amoebic meningoencephalitis caused by *Naegleria fowleri* (Seidel *et al.*, 1982). The inability of the drug to produce similar effect against *B. mandrillaris* suggests that although both amoeboe cause encephalitis, the treatment cannot be the same.

In this study, pentamidine isethionate was found to exhibit no activity against *Balamuthia* trophozoites. This finding is in contrast with a previous study which demonstrated the activity of pentamidine isethionate against trophozoites at 1 and 10 μg/ml (Schuster *et al.*, 1996). However, the variation in results can be explained by the different methodology used in each study. The study by Schuster *et al*. (1996) was based on growth inhibition of *B. mandrillaris* following drugs exposure. With this method, 82 % and 93 % growth inhibition were seen with 1 and 10 μg/ml of pentamidine isethionate respectively. Whereas the drug assay developed here was not quantitative but semi-quatitative, with a ‘yes’ or ‘no’ readout. Therefore, the assay was not able to distinguish the percentage of inhibition reported by Schuster *et al*. (1996) and results for 1 and 10 μg/ml would appear as 0% growth inhibition. In a study by Siddiqui and colleagues, which also used semi-quantitative methodology, 5 and 10

μg/ml of pentamidine isethionate would appear to have no anti-trophozoite or anti-cyst activity (Siddiqui *et al.*, 2008b).

Ketaconazole has been reported to prevent the formation of *B. mandrillaris* mature cysts and prevent the trophozoites from producing cytopathic effects on mammalian cell lines by interfering with ergosterol biosynthesis (Siddiqui *et al.*, 2007). In this study, sulcanazole, which is a related imidazole antifungal compound, was tested but no activity was exhibited against either the trophozoites or cysts. It has been well documented that azole-based drugs serve as antifungals by inhibiting the synthesis of ergosterol (Ghannoum *et al.*, 1999), which is the major element of the fungal cell membrane (Volkman, 2003). Thus results in death of the cell due to alteration in the membrane fluidity and integrity (Ghannoum *et al.*, 1999). Studies have shown that ergosterol is also a main sterol component in the *Acanthamoeba* membrane (Raederstorff *et al.*, 1985, Smith *et al.*, 1968). As *Balamuthia* is phylogenetically related to *Acanthamoeba* (Amaral Zettler *et al.*, 2000, Stothard *et al.*, 1998), it is possible that their membrane also contains ergosterol. Therefore, there is a possibility that the azole-based drugs would act on *Balamuthia* using a mechanism similar to the antifungal effect, by inhibiting the ergosterol biosynthesis. However, it has been reported that the precursors involved in ergosterol biosynthesis in *Acanthamoeba* (e.g. cycloartenol) are different from those of fungi (e.g. lanosterol) (Ghannoum *et al.*, 1999, Raederstorff *et al.*, 1985). Hence, there are differences in the structures of precursors and enzymes involved in the ergosterol biosynthesis in *Acanthamoeba* and fungi (Raederstorff *et al.*, 1985, Ghannoum *et al.*, 1999). It differences also exist between *B. mandrillaris* and fungal ergosterol biosynthesis, there they might affect the efficacy of the azole-based drug when used against *B. mandrillaris* and explain why the drugs were only amoebastatic and not amoebacidal to this amoeba.

Miltefosine which is commonly used as anticancer drug has previously been reported to cause lysis of *B. mandrillaris* trophozoites at concentrations above 40 µM (Schuster *et al.*, 2006a). The drug also has recently been used in combination with albendazole and fluconazole in the treatment of a BAE patient in Peru (Martinez *et al.*, 2010). However, these finding is opposed by the result obtained in this study which showed a MTAC of 250 µM. The precise reason for the variation is uncertain. However, the antimicrobial activity by Schuster *et al*. (2006) was reported based on the lack of large plaques caused by trophozoites lysis of a mammalian monolayer at 40 µM. Therefore, it could be possible that in their study low numbers of amoebae had survived but there were insufficient numbers to cause extensive lysis of the monolayer. It have been reported that factors such as strains used, duration of drug exposure and period of incubation of drug-treated *B. mandrillaris* in drug-free medium or on mammalian monolayers could cause variation in the drug assay results (Schuster *et al.*, 1996, Schuster *et al.*, 2006a). For example, out of six *B. mandrillaris* strains that were tested with miltefosine, five strains (V039, V188, V426, V433 and V451) and a strain (V194) showed minimal amoebicidal concentration of 40 µM and 75 µM respectively (Schuster *et al.*, 2006a). The absence of activity in most of the drugs tested in this study might indicate that they may not be effective when used as a single treatment but exhibit synergistic effects when use in combination with other drugs *in vivo*, as those observed in successful BAE treatments using mixtures of drugs (Martinez *et al.*, 2010, Deetz *et al.*, 2003). Due to inability to obtain other *B. mandrillaris* strains, the results obtained in this study might be limited against the strain ATCC 50209 used. Therefore, it would be advantageous if the drugs assays could be performed against other clinical and environmental strains to see whether similar results would be obtained.

In this study, the drug assay developed for *B. mandrillaris* cysts involves the use of *Acanthamoeba* as a food source for *Balamuthia*, rather than a mammalian cell monolayer, which have commonly been employed by other studies (Schuster *et al.*, 1996). As mentioned previously, *B. mandrillaris* does not readily feed on bacteria but will feed on mammalian cell lines and on other small soil amoebae, including *Acanthamoeba* (Matin *et al.*, 2006a, Schuster *et al.*, 2004c). The exact environmental niche of *B. mandrillaris* is currently unknown, however, its natural food source is not likely to be mammalian cells. Hence for this reason *Acanthamoeba* was selected as an alternative food source as this is more likely to be its natural prey in the environment. Here, a comparison was made by performing the drug assay with *Acanthamoeba* as the food source and also by inoculating the drug-treated *B. mandrillaris* cysts onto MA104 monolayers. As shown in the results, the MCCs using both types of food source were identical for amphotericin B, miltefosine and spiramycin. While the MCC for others drugs that were inoculated onto mammalian cells were two-fold lower than those MCC from drug assays using *Acanthamoeba* trophozoites. However, it is possible that when very low numbers of trophozoites are present, large plaques are not formed. Any plaques formed maybe too small to be seen by eye. It was observed that the *Balamuthia* trophozoites did not always cause visible clearing of the MA104 monolayer, resulting in the possibility of a false negative result. In contrast, drug assays using *Acanthamoeba* as the food source have the advantage that the results were easier to interpret. This is because the clear background of the wells, unlike those using MA104 monolayers allows excysted *B. mandrillaris* to be easily seen even in low numbers. In addition, the two amoebae showed large differences in morphology, making it straight forward to distinguish *Balamuthia* trophozoites from those of *Acanthamoeba*.

The significant activity showed by diminazene aceturate against both the trophozoite and cyst stages of *B. mandrillaris* is a new discovery, suggesting the prospective of a therapeutic agent or lead compound in the management of *Balamuthia* infections. Diminazene aceturate (Berenil®) is a veterinary drug used in the treatment of animal babesiosis and trypanosomiasis (Peregrine *et al.*, 1993). Even though not currently approved for human use, it has been commonly used in the treatment of African Trypanosomiasis in humans (Bacchi, 2009, Peregrine *et al.*, 1993) eventhough neurological side-effects have been reported with the use of the drug in treating a patient with babesiosis (Ruebush *et al.*, 1979). Perhaps, in order to minimise the undesirable side-effects, the drug should be prescribed as an individually licensed drug. The exact mechanism of action of dimiazene aceturate is unknown but previous studies with trypanosomes have demonstrated that related diamidine compounds intercalate with DNA, inhibiting replication (Bacchi, 2009, Peregrine *et al.*, 1993). In addition, studies with *Acanthamoeba* have shown that diamidine compounds inhibit polyamine biosynthesis and disrupt the plasma membrane lipid bilayer through a detergent-like action (Byers *et al.*, 1991, Perrine *et al.*, 1995). Whether diminazene aceturate acts in a similar way against *B. mandrillaris* is not known, but merits further investigation. Animal models using normal immunocompetent Balb/c and severe combined immunodeficient mice (SCID) have been described for studying Balamuthia amoebic encephalitis following intranasal or intraperitoneal inoculation with *B. mandrillaris* trophozoites and cysts (Janitschke *et al.*, 1996). Death of mice was only seen following infection by the intranasal route when 70% SCID and 10% Balb/c mice died (Janitschke *et al.*, 1996). *B. mandrillaris* trophozoites and cysts were detected in brains or lungs sections by microscopy following haematoxylin-eosin staining and immunohistochemistry with *B. mandrillaris* polyclonal antibodies (Janitschke *et al.*,

1996). Inoculation of brain samples from two of the 12 mice onto monkey kidney cell monolayers resulted in growth of *B. mandrillaris* (Janitschke *et al.*, 1996). Such a model could be applied for testing the activity of a candidate drug that was shown to be effective against *B. mandrillaris* by *in vitro* drug assays.

Although BAE is a rare disease, the outcome is invariably fatal if not treated. A few patients have survived the infection following treatment with a mixture of drugs including pentamidine isethionate, sulfadiazine, azithromycin/clarithromycin, flucytosine and fluconazole, despite *in vitro* drug efficacy studies performed previously with these agents showing in some cases only moderate activities or only amoebastatic against the amoeba (Deetz *et al.*, 2003, Jung *et al.*, 2004, Schuster *et al.*, 1996, Schuster *et al.*, 2008a). The findings of this study indicate that diminazene aceturate has superior efficacy against *B. mandrillaris* and may offer an improved prognosis if used in therapy for this disease.

8 General discussion

The major obstacle in extracting DNA directly from environmental samples is the co-existence of humic substances, which are characterised as having similar size and charge to DNA (Holben, 1994). The presence of humic substances, such as fulvic acid, humic acid and humin, in DNA extracts can act as a potent inhibitor for PCR and enzymatic digestions (Young *et al.*, 1993, Paul *et al.*, 1996, Yeates *et al.*, 1997). Therefore, it is extremely important to use a suitable direct DNA extraction method for environmental samples to avoid false negative PCR results. It has been suggested that a good direct DNA extraction would produce: 1) high molecular weight DNA; 2) DNA that is free from PCR inhibitors and 3) adequate lysis of microorganisms within the sample (Yeates *et al.*, 1997). In this study, a direct DNA extraction method for environmental samples was developed using a combination of the UNSET-PEG method and purification using a ZR soil microbe kit (Zymo Research Corp.). This method could be a significant tool in the study of the ecology of free-living amoebae. Although the ecology of *Naegleria* and *Acanthamoeba* can be investigated by isolation and culture using the conventional non-nutrient agar plates seeded with *E. coli*, the application of molecular methods also can be very useful. However, it is in the investigation of amoebae which are not easily cultured (or have not yet been cultured) that molecular methods find a prominent place. It is in this situation that the method developed here for DNA extraction directly from environmental samples (see Chapter 4) could find its greatest application for studying the ecological distribution of *B. mandrillaris*. The DNA extraction method developed in this present study is not only suitable to be used for soil samples but can also be applied for various types of water samples, including those containing sediments, algae, small plants or stones. The DNA extracts are free

from PCR inhibitors and suitable to be used for the detection of *B. mandrillaris* which is difficult to isolate using the NNA-*E. coli* plates.

A specific and sensitive *B. mandrillaris* nested PCR was developed in this study for the detection of *B. mandrillaris* from environmental samples. The successful application of the developed direct DNA extraction method and *B. mandrillaris* nested PCR in detecting the presence of the amoeba in soil and water samples showed their usefulness as a new approach for studying the ecological distribution of *B. mandrillaris*. The combination of the developed methods may improve the current detection method which relies on growth of the amoeba following culturing of environmental materials on NNA-*E. coli* plates (Dunnebacke *et al.*, 2003, Schuster *et al.*, 2003, Niyyati *et al.*, 2009). This is because the detection of *B. mandrillaris* using the conventional method is not always successful or a lengthy process due to requirement of frequent microscopic observations and subculturing (Rideout *et al.*, 1997, Dunnebacke *et al.*, 2004). The detection of *B. mandrillaris* DNA using the developed methods particularly in >90% soils of Southern California and >50% water samples collected from a thermally enriched river in France clearly suggests the preference of the amoeba for warmer regions or habitats. Further study of a wider range of samples from temperate and warmer regions is required. The availability of the developed methods will make this an easier proposition. The DNA extraction method and nested PCR developed here allows fast results to be obtained and therefore it is suitable to be used for screening large numbers of environmental samples and therefore will allow more ecological studies of *B. mandrillaris* to be performed.

Balamuthia mandrillaris is a slow growing amoeba and therefore can easily be overgrown by other free-living amoebae in cultures (Schuster *et al.*, 1996, Schuster *et al.*, 2003). To this end, the production of poly- and monoclonal antibodies against the

amoeba was undertaken in the belief that they would aid in the isolation of *B. mandrillaris* from environmental samples when used in immunomagnetic separation using Dynabeads (Dynal, Oslo, Norway). It was revealed in Chapter 6 that the immunomagnetic separation was not suitable to be used for isolation of *B. mandrillaris* trophozoites due to toxicity of the chemicals and inability of the trophozoites to survive the separation process. In contrast, *B. mandrillaris* cyst stage was shown to be able to survive the harsh separation process but the isolated cysts were often contaminated with fungi thus hindering further culturing on mammalian cells. The findings suggested the prospect of use of the immunomagnetic separation technique in the isolation of *B. mandrillaris* cysts from environmental samples if the problem with fungal contamination can be resolved. The monoclonal antibodies developed in this study have limited application in immunomagnetic separation due to their poor reactivity against *B. mandrillaris* cysts. However, the specificity of the monoclonal antibodies against the trophozoite stage showed their usefulness as a potential diagnostic tool, especially for confirmation of the presence of *B. mandrillaris* trophozoites in clinical specimens. Currently, the confirmation of BAE cases are performed by immunofluorescence assays of clinical samples with *B. mandrillaris* polyclonal antibodies (Visvesvara *et al.*, 1993). Although polyclonal antibodies are fast to produce, they have several disadvantages, such as being prone to batch variability and lack of specificity (Lipman *et al.*, 2005) as shown by the ability of the *B. mandrillaris* polyclonal antibodies made in this study to react with *Acanthamoeba* and *Naegleria* trophozoites. In this situation, the monoclonal antibodies developed in this study offer some advantages due to their consistency and lack of cross-reactivity against *Acanthamoeba* and *Naegleria*. Future work may include determining the value of the monoclonal antibodies for the detection of *B. mandrillaris*

trophozoites in brain and skin biopsies when use in immunological methods such as immunofluorescence assay.

The success of treatment for *B. mandrillaris* diseases is dependent on early diagnosis and initiation of appropriate drug regimen (Deetz *et al.*, 2003, Jung *et al.*, 2004). The choice of drugs to be used in treating infected individuals has been mainly based on *in vitro* drug studies using clinical *B. mandrillaris* strains (Schuster *et al.*, 2008a, Schuster *et al.*, 1996) but these methods have practical deficiencies. In this study, improved *in vitro* drug assays for *B. mandrillaris* trophozoites and cysts were developed using BM3 axenic medium removing the need for further confirmation of the viability of the drug-treated amoeba using mammalian monolayers. In addition, the improved drug assays are less laborious because the efficacy of tested drugs is assessed by the presence or absence of normal branching trophozoites following transfer to drug-free culture medium rather than percentage of growth inhibition which requires counting of amoeba at several time intervals as employed in previous studies (Schuster *et al.*, 1996, Schuster *et al.*, 2006a). In the improved drug assay for *B. mandrillaris* cysts, the use of *Acanthamoeba* as the food source made the results easier to interpret due to the distinct morphological differences between the two amoebae and clear background of the wells which allows easy detection of the excysted *B. mandrillaris* even in low numbers. In contrast, the drug-treated cysts that were transferred onto mammalian monolayers, as was done in previous assays, could lead to false-negative results especially when the excysted *B. mandrillaris* were present but did not cause visible clearing of the monolayer. Using these improved drug assays, diminazene aceturate was shown to have high efficacy, not only against *B. mandrillaris* trophozoites, but also the cysts. The application of the drug in the treatment of Balamuthia amoebic encephalitis or skin lesions using animal models may be worth investigating. Perhaps the drug may also be

considered as a lead compound for a medicinal chemistry programme to improve its efficacy and reduce toxicity for use in humans.

A minor part of the study in this thesis involved the detection of *N. fowleri* from environmental samples using a combination of the developed DNA extraction method and *N. fowleri* one-step nested PCR. The advantage of the *N. fowleri* one-step nested PCR is the reaction is performed in a single tube, which is less laborious and minimise the risk of cross-contamination that can be encountered in conventional two-step nested PCR (Reveiller *et al.*, 2002) especially when involving large numbers of samples. Hence, the developed detection method may allow rapid detection of *N. fowleri* from environmental samples to be performed and overcome the false-negative problem due to overgrown by other thermophilic species such as *N. lovaniensis* that is commonly encountered with detection based on primary culturing on NNA-*E. coli*.

Overall, the developed direct detection method using a combination of direct DNA extraction method and *B. mandrillaris* nested PCR may allow greater understanding of the ecological and geographical distribution of *B. mandrillaris* and risk of human infections. The production of specific monoclonal antibodies and identification of diminazene aceturate as a potential drug may provide better diagnosis and treatment of *B. mandrillaris* diseases thus reducing fatal outcomes. The improved *in vitro* drug assays also may permit many potential drugs to be tested in the future. Finally, utilisation of the direct DNA extraction method and *N. fowleri* one-step nested PCR for rapid detection of *N. fowleri* from the environment may allow the implementation of preventative public health measures for avoiding cases of human infection and also to assist ecological studies.

9 Appendices

9.1 Appendix 1 (Preparation of reagents and culture media)

Carbonate coating buffer, pH 9.6

Sodium hydrogen carbonate ($NaHCO_3$)	1.465 g
Sodium carbonate (Na_2CO_3)	0.795 g

Add 400 ml of nanopure water and adjust pH to 9.6 with 1M HCl. Top up with nanopure water to a final volume of 500 ml.

Chelex extraction solution

Chelex-100 resin (Bio-Rad Lab. Ltd., Hertfordshire, UK)	2 g
Triton X 100 (10% v/v in H_2O)	200 µl
Tris-HCl, pH 8.0 (10 mM)	to 20 ml

Autoclave at 121°C for 15 min and store at room temperature.

DABCO (1, 4-diazabicyclo [2.2.2] octane)

DABCO	0.233 g
Tris-HCl (1M, pH 8.0)	200 µl
Nanopure water	800 µl
Glycerol, 86%	9 ml

Dissolve the components by warming to 70°C. Vortex and aliquot in 2 ml tubes and keep at -20°C until use.

Dulbecco A phosphate buffered saline (DPBS)

DPBS tablets (Oxoid Ltd., Hampshire, UK)	5 tablets
Nanopure water	500 ml

Autoclave at at 121°C for 15 min and store at room temperature.

EDTA $Na_2.2H_2O$ (0.5M, pH 8)

EDTA di-sodium salt ($C_{10}H_{14}N_2Na_2O_8.2H_2O$)	186.1 g
Nanopure water	800 ml

The solution was stirred vigorously on a magnetic stirrer. The pH was then adjusted to 8.0 with NaOH pellets. The EDTA di-sodium salt does not dissolve until the pH of the

solution is pH 8.0. Nanopure water was added to 1 L, autoclaved and kept at room temperature until used.

LB (Luria-Bertani) broth*

Luria Bertani powder (BD Biosciences, Oxford, UK)	5 g
Nanopure water	200 ml

Autoclave at 121°C for 15 min. Store at RT for use within 6 months.

* To prepare LB agar add 1.5% (w/v) to the broth prior to autoclave.

L-methionine (5 mg/ml)

L-methionine	500 mg
Nanopure water	100 ml

Autoclave at 121°C for 15 min. Aliquot in 20 ml volumes and store at -20°C for use within 12 months. Thawed aliquot is stored at 4°C and use within 1 month.

Non-nutrient agar (NNA)

¼ strength Ringer's tablet (Oxoid Ltd., Hampshire, UK)	1 tablet
Nanopure water	500 ml
Agar (Lab M Ltd., Lancashire, UK)	7.5 g

Autoclave at 121°C for 15 min. Let to cool until 50°C. Mix thoroughly by gentle shaking and pour to onto Petri dishes. Allow to solidify at RT and keep at 4°C for use within 2 weeks.

Sabouraud agar

Sabouraud agar	32.5 g
Nanopure water	500 ml

Autoclave at 121°C for 15 min. Let to cool until 50°C. Mix thoroughly by gentle shaking and pour to onto Petri dishes. Allow to solidify at RT and keep at 4°C for use within 2 weeks.

$TE_{0.1mM}$ buffer

Tris-HCl, (1M, pH 8)	10 mM
EDTA $Na_2.2H_2O$ (0.5 M, pH 8)	0.1 mM

Tris-acetate-EDTA buffer (10× TAE)

Tris base	48.4 g
Glacial acetic acid	11.4 ml
EDTA Na2.2H2O (0.5M, pH 8)	20 ml
Nanopure water	to 1litre

Adjust to pH 7.9 if necessary. Autoclave at 121°C for 15 minutes. Use at 1× concentration for agarose gel electrophoresis.

Tryptone Soy Agar

Tryptone soy agar	40 g
Nanopure water	up to 1 litre

Autoclave at 121°C for 15 min. Let to cool until 50°C. Mix thoroughly by gentle shaking and pour to onto Petri dishes. Allow to solidify at RT and keep at 4°C for use within 2 weeks.

UNSET lysis buffer (Hugo *et al.*, 1992)

Urea	48 g (8M)
Sarkosyl (20%)	10 ml (2%)
NaCl (5 M)	3 ml (0.15 M)
EDTA $Na_2.2H_2O$ (0.5 M, pH 8)	0.2 ml (0.001 M)
Tris pH 7.5(1 M)	10 ml (0.1 M)
Nanopure water	to 100 ml

Original recipe used SDS (sodium dodecyl sulphate). Filter sterilise and store at room temperature

Vitamin B_{12} (100 µg/ml)

Vitamin $_{B12}$	10 mg
Nanopure water	100 ml

Autoclave at 121°C for 15 min. Aliquot in 10 ml volumes and store at -20°C for use within 12 months. Thawed aliquot is stored at 4°C and use within 1 month.

0.05% (v/v) T-DPBS

Tween 20	250 µl
DPBS	500 ml

0.05% (v/v) T-DPBS + 1% (w/v) skimmed milk

Skimmed milk powder	0.5 g
0.05% (v/v) T-DPBS	50 ml

0.05% (v/v) T-DPBS + 5% (w/v) skimmed milk

Skimmed milk powder	2.5 g
0.05% (v/v) T-DPBS	50 ml

0.18M Sulphuric acid (H_2SO_4)

1M H_2SO_4	18 ml
Nanopure water	to 100 ml

0.3% (v/v) T-DPBS

Tween 20	1.5 ml
DPBS	500 ml

0.3% (v/v) T-DPBS + 3% (w/v) skimmed milk

Skimmed milk powder	1.5 g
0.05% (v/v) T-DPBS	50 ml

1 M HCl solution

Concentrated HCl (37.5%)	8.2 ml
Nanopure water	to 100 ml

1M Sodium hydroxide (NaOH)

NaOH	4 g
Nanopure water	100 ml

1M Sulphuric acid (H_2SO_4)

H_2SO_4	5.56 ml
Nanopure water	to 100 ml

5% (w/v) neutralised liver digest in HBSS

Neutralised liver digest	5 g
Nanopure water	90 ml

Autoclave at 121°C for 15 min. Aseptically add 10 ml of HBSS (10×)

30% (w/v) PEG 6000-1.6 M NaCl

5 M NaCl (Sodium chloride)	32 ml
PEG 6000	30 g
Nanopure water	to 100 ml

¼ strength Ringer's solution

¼ strength Ringer's tablet (Oxoid Ltd., Hampshire, UK)	1 tablet
Nanopure water	500 ml

Autoclave at 121°C for 15 min. Store at room temperature for use within 6 months.

9.2 Appendix 2 (Clipped files of DNA sequences)

> SC09-02 (*B. mandrillaris*)

TGTGGAGAAGGGCAGGGACGTAATCAACGCGAGCTGATGACTCGCGCTTAC
TAGGAATTCCTCGTTGAAGATTAACAATTACAATAATCTATCCCCATCACGA
TTAAATTTCACAAGATTACCCAGACCTTTCGGCCAAGGTGTGGA

> SC09-03 (*B. mandrillaris*)

AGAAAGGGCAGGGACGTAATCAACGCGAGCTGATGACTCGCGCTTACTAGG
AATTCCTCGTTGAAGATTAACAATTACAATAATCTATCCCCATCACGATTAA
ATTTCACAAGATTACCCAGACCTTTCGGCCAAGGTGTGGATAATCTT

> SC09-04 (*B. mandrillaris*)

AGGGACGTAATCAACGCGAGCTGATGACTCGCGCTTACTAGGAATTCCTCG
TTGAAGATTAACAATTACAATAATCTATCCCCATCACGATTAAATTTCACAA
GATTACCCAGACCTTTCGGCCAAGGTGTGGATAATCTTGTGAAATTTAATCG
TGATGGGGATAGATTATTGTAATTGTTAATCTTCAACGAGGAATTCCGAGTG
AGCGCGAGTCATCAGCTCGCGTTGATTACGTCCGTGCCCTTTGTAC

> SC09-05 (*B. mandrillaris*)

CAACGCGAGCTGATGACTCGCGCTTACTAGGAATTCCTCGTTGAAGATTAAC
AATTACAATAATCTATCCCCATCACGATTAAATTTCACAAGATTACCCAGAC
CTTTCGGCCAAGGTGT

> SC09-07 (*B. mandrillaris*)

GGGCGGTGTGTAGAAAGGGCAGGGACGTAATCAACGCGAGCTGATGACTC
GCGCTTACTAGGAATTCCTCGTTGAAGATTAACAATTACAATAATCTATCCC
CATCACGATTAAATTTCACAAGATTACCCAGACCTTTCGGCCAAGGTGTGGA
TAATCTGGATAATCTTGT

> SC09-09 (*B. mandrillaris*)

AGCGACGGGCGGTGTGTAGAAAGGGCAGGGACGTAATCAACGCGAGCTGA
TGACTCGCGCTTACTAGGAATTCCTCGTTGAAGATTAACAATTACAATAATC
TATCCCCATCACGATTAAATTTCACAAGATTACCCAGACCTTTCGGCCAAGG
TGTGGATAATCTTGTGAAATTTAATCGTGAT

> SC09-10 (*B. mandrillaris*)

GGCAGGGACGTAATCAACGCGAGCTGATGACTCGCGCTTACTAGGAATTCC
TCGTTGAAGATTAACAATTACAATAATCTATCCCCATCACGATTAAATTTCA
CAAGATTACCCAGACCTTTCGGCCAAGGTGTGGATAATCT

> SC09-11 (*B. mandrillaris*)

GACGGGCGGTGTGTAGAAAGGGCAGGGACGTAATCAACGCGAGCTGATGA
CTCGCGCTTACTAGGAATTCCTCGTTGAAGATTAACAATTACAATAATCTAT
CCCCATCACGATTAAATTTCACAAGATTACCCAGACCTTTCGGCCAAGGTGT
GGATAATCTTGTGAAATTTAATCGTGATGGGGATAGATTATTGTAATTGTTA
ATCTTCAACGAGGAATTCCTAGTAAGCGCGAGTCATCAGCTCGCGTTGATTA
CGTCCCTGCCCTTTGTACACACCGCCCGTCGCTTCTAC

> SC09-12 (*B. mandrillaris*)

GCGACGGGCGGTGTGTAGAAAGGGCAGGGACGTAATCAACGCGAGCTGAT
GACTCGCGCTTACTAGGAATTCCTCGTTGAAGATTAACAATTACAATAATCT
ATCCCCATCACGATTAAATTTCACAAGATTACCCAGACCTTTCGGCCAAGGT
GTGGATAATCTTGTGAAATTTAATCGTGATGGGGATAGATTATTGTAATTGT
TAATCTTCAACGAGGAATTCCTAGTAAGCGCGAGTCATCAGCTCGCGTTGAT
TACGTCCCTGCCCTTTGTACACACCGCCCGGCGCTCC

> PT09-07 (*B. mandrillaris*)

AGCGACGGGCGGTGTGTAGAAAGGGCAGGGACGTAATCAACGCGAGCTGA
TGACTCGCGCTTACTAGGAATTCCTCGTTGAAGATTAACAATTACAATAATC
TATCCCCATCACGATTAAATTTCACAAGATTACCCAGACCTTTCGGCCAAGG
TGTGGATAATCTTGTGAAATTTAATCGTGATGGGGATAGATTATTGTA

> FW08-19 (*B. mandrillaris*)

AGCGACGGGCGGTGTGTAGAAAGGGCAGGGACGTAATCAACGCGAGCTGA
TGACTCGCGCTTACTAGGAATTCCTCGTTGAAGATTAACAATTACAATAATC
TATCCCCATCACGATTAAATTTCACAAGATTACCCAGACCTTTCGGCCAAGG
TGTGGATAATCTT

> FW08-20 (*B. mandrillaris*)

GAGCGACGGGCGGTGTGTAGAAAGGGCAGGGACGTAATCAACGCGAGCTG
ATGACTCGCGCTTACTAGGAATTCCTCGTTGAAGATTAACAATTACAATAAT
CTATCCCCATCACGATTAAATTTCACAAGATTACCCAGACCTTTCGGCCAAG
GTGTGGATAATCTTGTG

> FW08-32 (*B. mandrillaris*)

GAGCGACGGGCGGTGTGTAGAAAGGGCAGGGACGTAATCAACGCGAGCTG
ATGACTCGCGCTTACTAGGAATTCCTCGTTGAAGATTAACAATTACAATAAT
CTATCCCCATCACGATTAAATTTCACAAGATTACCCAGACCTTTCGGCCAAG
GTGTGGATAATCTTGTGTAGTCTA

> **FW08-36 (*B. mandrillaris*)**

AGCGACGGGCGGTGTGTAGAAAGGGCAGGGACGTAATCAACGCGAGCTGA
TGACTCGCGCTTACTAGGAATTCCTCGTTGAAGATTAACAATTACAATAATC
TATCCCCATCACGATTAAATTTCACAAGATTACCCAGACCTTTCGGCCAAGG
TGTGGATAATCTTGTG

> FW08-27 (*N. fowleri*)

TTGTCAATCGAGCATGACTTGCTTTAACGAATGCTTGTAAAGTGACAAGTTG
TGATAATATCTTTCTCCATTGGATTTGAGAACAATGGTGTCGTCGAAGAAGA
GACTGCATTTGAAGAATCTGTTGGAGTGAATGTTCCACTTCTTGGCGAGCTT
GTGTGGAAAGGAAGCACATGCTCACTACTTGAACGAGTGATTTCAATTTGA
AATAATGGTCTCTTTGTTGTTTTGCACGAAATGCAGATTGTAGGGTACGAAT
CGCTCCAAGTGAGAGAAGTAATTGGTTGGAAGTCGTTAACTGTCTGAATCT
AGCTTGAAGTTGAAGAGACTTTTCCAAGAGTTGATGTTTTTCTTTTCTTACTT
TCTTTGATCTGATCCATGACTGAATGAGTGTGATGACATTCCTGCTCCCC

> FW08-28 (*N. fowleri*)

AGAGTCTGCCTTTGAGGACACTCTGCACCTGTGAAAGAGTATTCAGAGAAT
GTTCAATTGTCAATCGAGCATGACTTGCTTTAACGAATGCTTGTAAAGTGAC
AAGTTGTGATAATATCTTTCTCCATTGGATTTGAGAACAATGGTGTCGTCGA
AGAAGAGACTGCATTTGAAGAATCTGTTGGAGTGAATGTTCCACTTCTTGGC
GAGCTTGTGTGGAAAGGAAGCACATGCTCACTACTTGAACGAGTGATTTCA
ATTTGAAATAATGGTCTCTTTGTTGTTTTGCACGAAATGCAGATTGTAGGGT
ACGAATCGCTCCAAGTGAGAGAAGTAATTGGTTGGAAGTCGTTAACTGTCT
GAATCTAGCTTGAAGTTGAAGAGACTTTTCCAAGAGTTGATGTTTTTCTTTT
CTTACTTTCTTTGATCTGATCCATGACTGAATGAGTGTGATGACATTCCTGA
GCCACTGAC

> FW08-68 (*N. fowleri*)

GTAAGAAAAGAAAAACATCAACTCTTGGAAAAGTCTCTTCAACTTCAAGCT
AGATTCAGACAGTTAACGACTTCCAACCAATTACTTCTCTCACTTGGAGCGA
TTCGTACCCTACAATCTGCATTTCGTGCAAAACAACAAAGAGACCATTATTT
CAAATTGAAATCACTCGTTCAAGTAGTGAGCATGTGCTTCCTTTCCACACAA
GCTCGCCAAGAAGTGGAACATTCACTCCAACAGATTCTTCAAATGCAGTCTC
TTCTTCGACGACACCATTGTTCTCAAATCCAATGGAGAAAGATATTATCACA
ACTTGTCACTTTACAAGCATTCGTTAAAGCAAGTCATGCTCGATTGACAATT
GAACATTCTCTGAATACTCTTTCACAGGTGCAGAGTGTCCTCAAAGGCAGAC
TTTCTCAACTCTACTTGTCTCGTGACTTGCAACGAGAACTCATTCA

> FW08-72 (*N. fowleri*)

GTAAGAAAAGAAAAACATCAACTCTTGGAAAAGTCTCTTCAACTTCAAGCT
AGATTCAGACAGTTAACGACTTCCAACCAATTACTTCTCTCACTTGGAGCGA
TTCGTACCCTACAATCTGCATTTCGTGCAAAACAACAAAGAGACCATTATTT
CAAATTGAAATCACTCGTTCAAGTAGTGAGCATGTGCTTCCTTTCCACACAA
GCTCGCCAAGAAGTGGAACATTCACTCCAACAGATTCTTCAAATGCAGTCTC
TTCTTCGACGACACCATTGTTCTCAAATCCAATGGAGAAAGATATTATCACA
ACTTGTCACTTTACAAGCATTCGTTAAAGCAAGTCATGCTCGATTGACAATT
GAACATTCTCTGAATACTCTTTCACAGGTGCAGAGTGTCCTCAAAGGCAGAC
TTTCTCAACTCTACTTGTCTCGTGACTTGCAACGAGAAAC

9.3 Appendix 3 (DNA sequence of cloned fragment of *N. fowleri* strain MCM, pUC PB2.3)

5’agagtcgagaaagtgtcattactttgcaagcgaagatgagacactatttgtgttccaaagctttgaatgcaagtaagaaagct
atcgaatggattcaagcaaaaagtaagagttcattaacatacaaacaatcacaacaatccctccaaagaatcctcactctgcaa
gctctgatgagatccttccacgagaagcatgtccaacgccaagacttacatcgaatatttatattgaactctctcattagatccaat
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caatcatgcctttcttcggctcgcatggcacaagcaatgattcgaggaaggcaagatagacattccaaaaatggaaaccaaca
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caatccatgttcatgtcaacacactgcataaaacacatgacccgtgacaggaatgtcatcacactcattcagtcatggatcagat
caaagaaagtaagaaaagaaaaacatcaactcttggaaaagtctcttcaacttcaagctagattcagacagttaacgactttcaa
ccaattacttctctcacttggagcgattcgtaccctacaatctgcatttcgtgcaaaacaacaaagagaccattatttcaaattgaa
atcactcgttcaagtagtgagcatgtgcttcctttccacacaagctcgccaagaagtggaacattcactccaacagattcttcaaa
tgcagtctcttcttcgacgacaccattgttctcaaatccaatggagaaagatattatcacaacttgtcactttacaagcattcgttaa
agcaagtcatgctcgattgacaattgaacattctctgaatactctttcacaggtgcagagtgtcctcaaaggcagactttctcaact
ctacttgtctcgtgacttgcaacgagtactcattctacaagcactcattcaatctcgtgcatgtcacatcaagtggcgtgcactcaa
ggggcgagcacgccatttccgagagaaaatgaatgacatttctgtcatgcaaggtttcattcgaggatttctctatcggagagat
gatttattatacgtgagtcgtcacaagaatgcagatactggctcgagtgtgatggttccatcgacttcagacattttacgtcaactc
tatgacgaggaagatgaatattatcaagcagcacattctgaagaagacgaaaccaatgacaacaacaaggatgagaccattc
gtgaaagtttacaaaaactaaatctaaaagaatgcactcaaatacttggcgtgaaagtttacaaaaactaaatctaaaagaatgc
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acactcttcttacaaaagaaagaaacacaagatgatcatttcaataagccttccacgagtagtgttcaatccctattgatggaatct
tcttcacaacatttccacaaaaaatcacaatccattacatctgttttgaccaatcgagatccgt-3’

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