Interaction of Free-living protozoa with water-borne human pathogenic viruses and protection from disinfection

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By

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Abstract

Acanthamoeba causes Granulomatous Amoebic Encephalitis (GAE) and Amoebic Keratitis (AK) in humans and in its cystic form is resistant to extreme environmental conditions. Both human pathogenic water-borne viruses and free-living protozoa share the same aquatic environment. This study set out to test the ability of both *Acanthamoeba* and *Tetrahymena* to internalise and protect enteric viruses; coxsackievirus (B3, B5), poliovirus (PV) and rotavirus (RV) following co-culture. Viral uptake was assessed by infection of cultured mammalian cells, by indirect immunofluorescence (IF), and by reverse transcriptase-polymerase chain reaction (RT-PCR). The results showed that none of the free suspended viruses were internalised in *Acanthamoeba* or *Tetrahymena.* However, both coxsackievirus B3N and rotavirus Wa could be detected within *Acanthamoeba* by IF and confirmed by RT-PCR when the amoebae were co-cultured (fed) with virally infected mammalian cells. The co-cultured amoeba was allowed to encyst but following this procedure no viruses were detected either by cell culture or RT-PCR.

In a second series of experiments, the efficacy of solar disinfection (SODIS) against viruses either alone or when co-cultured with *Acanthamoeba* was assessed. SODIS reduced the viral infectivity by over $3\log_{10}$ after 1 h for CVB3N and over $2\log_{10}$ for PV after 2 h. Repeating these experiments in the presence of riboflavin, a $6\log_{10}$ reduction was observed for CVB3N after 1 h of light exposure and $6\log_{10}$ after 6 h for all other viruses tested.

The results suggest that *Acanthamoeba* does not internalise or protect viruses in suspension. However, if a virus is located with an infected mammalian cell then it may be internalised; a new potential mechanism for virus dissemination in the environment. Secondly, solar disinfection is an effective treatment method for water contaminated with viruses which is further enhanced by the addition of riboflavin. This study provides a practical example of low technology methods which could be utilised to provide safe drinking water in various circumstances.

Statement of Originality

This accompanying thesis submitted for the degree of PhD thesis is based on work conducted by the author at the University of Leicester mainly during the period between January 2005 and December 2010.

All the work recorded in this thesis is original unless otherwise acknowledge in the text or by references.

None of the work has been submitted for another degree in this or any other university.

Mohammad Alotaibi

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Abbreviations

	Abbreviation	definition
1	µl	Microlitre
2	µm	Micrometre
3	*A. castellanii*	*Acanthamoeba castellanii*
4	*A. polyphaga*	*Acanthamoeba polyphaga*
5	A.K.	*Acanthamoeba* keratitis
6	ATCC	American type culture collection
7	BSA	Bovine serum albumin
8	bp	Base pair
9	c.f.u	Colony forming unit
10	CPE	Cytopathic effect
11	°C	Degree centigrade
12	Car	Coxsackievirus-adenovirus receptor
13	CO_2	Carbon dioxide
14	CSF	Cerebrospinal fluid
15	CVB3N	Coxsackievirus B3 Nancy
16	CVB5F	Coxsackievirus B5 Faulkner
17	DAF	Decay accelerating factor
18	DMEM	Dulbecco's Modified Eagle Medium
19	DNA	Deoxyribonucleic acid
20	PBS	Dulbecco's Phosphate Buffered Saline
21	dsDNA	Double stranded Deoxyribonucleic acid
22	dsRNA	Double stranded Ribonucleic acid
23	*E. coli*	*Escherichia coli*
24	EDTA	Ethylenediaminetetraacetic acid
25	EM	Electron microscope
26	ER	Endoplasmic reticulum
27	FBS	Foetal bovine serum
28	FITC	Fluorescein Isotiocianate
29	*g*	Gravity
30	GAE	Granulomatous Amoebic Encephalitis
31	GLV	*Giardia Lamblia* Virus
32	H_2O	Water
33	H_2O_2	Hydrogen peroxide
34	HEK	Human Embryonic Kidney 293 cells
35	HEp-2	Contaminant of Hela (Henrietta Lacks) cells (Cancer cells of cervix)
36	IFA	Immunofluorescence assay
37	ICAM-1	Intercellular adhesion molecule 1
38	IgG	Immunoglobulin G
39	kbp	Kilo-base-per
40	*L. monocytogenes*	*Listeria monocytogenes*
41	m^2	square metre
42	MA104	African Green Monkey kidney cells
43	MEME	Minimum Essential Medium Eagle

44	$MgCl_2$	Magnesium chloride
45	ml	millilitre
46	MRSA	Methicillin-resistant *staphylococcus aurous*
47	*N. gruberi*	*Naegleria gruberi*
48	Nm	Nanometre
49	NS (#)	Non-structural protein (#)
50	PBS	Phosphate buffer saline
51	p.f.u.	Plaque forming unit
52	PV-2	Poliovirus type 2
53	RAPD	Random Amplification of Polymorphic DNA
54	RFLP	Restriction fragment length polymorphism
55	RI	Replicative intermediates
56	RNA	Ribonucleic acid
57	rRNA	Ribosomal ribonucleic acid
58	RPMI 1640	Roswell Park Memorial Institute medium
59	rRNA	Ribosomal Ribonucleic acid
60	RT-PCR	Reverse transcriptase-polymerase chain reaction
61	Rv Wa	Rotavirus Wa
62	SCGYEM	serum-casein-glucose-yeast extract medium
63	SODIS	Solar disinfection
64	ssDNA	Single stranded Deoxyribonucleic acid
65	+ssRNA	positive-sense single stranded ribonucleic acid
66	-ssRNA	negative-sense single stranded ribonucleic acid
67	ssRNA	Single stranded Ribonucleic acid
68	spp.	Species
69	*T. pyriformis*	*Tetrahymena pyriformis*
70	TCID	Tissue culture infective dose
71	TVV	*Trichomonous vagainalis* virus
72	USA	United States of America
73	UV	Ultraviolet
74	*V. cholerae*	*Vibrio cholerae*
75	VLP	virus like particles
76	VP (#)	Viral protein (#)
77	W	Watt

Chapter One: Introduction

Introduction

1.0 Purpose of the study

The free-living protozoan *Acanthamoeba* is virtually ubiquitous in the environment, particularly aquatic environments. There it can encounter a wide range of other microorganisms including bacteria, other protozoa and viruses where it will naturally interact with them. Many of these interactions involve predation upon other microorganisms, using them as a food source. Several microorganisms can, however, survive internalisation within the amoeba and form an association with them, such as *Legionella* and *Vibrio cholerae*. Internalisation of viruses by amoeba may also occur in nature. It has been suggested that *Acanthamoeba* could act as a potential reservoir of adenoviruses in the environment and be a vehicle for their transmission (Mattana *et al.,* 2006). Previous studies are limited but have suggested that internalisation of enteric viruses by *Tetrahymena pyriformis* could serve to prolong enterovirus survival of municipal sewage water. In addition, coxsackievirus B3 has been reported to be adsorbed onto the surface of *A. castellanii* cells and then to accumulate inside the cells following co-culture (Mattana *et al.,* 2006). The objective of the current is to provide new information on the implications of the association between free-living aquatic protozoa and water-borne pathogenic virus survival and dissemination. In this study a thorough investigation of the possibility of internalisation by free-living protozoa of a number of enteric viruses including coxsackievirus, poliovirus and rotavirus which are known to have a waterborne route of transmission was undertaken. This investigation involved simultaneous co-culturing of protozoa and viruses, to determine if the pathogenic viruses are internalised. A number of methods were used to determine whether virus internalisation had or had not occurred including immunofluorescence microscopy, amplification of viral nucleic acid sequences and through in vitro cell culture. Both freely suspended viruses and mammalian cells infected with viruses were used in the study, to investigate alternative routes for virus internalisation by protozoa.

The coxsackievirus B3 was used as a positive control, to replicate the experiment of Mattana *et al.,* (2006) who reported that CVB3N was internalised in *A. castellanii*. CVB5F was used because it is reported to be resistant to chlorine disinfection (Payment *et al.,* 1985). In addition, poliovirus was used because the World Health Organisation

(WHO) has made an ongoing eradication campaign great effort to eradicate it worldwide (WHO, 2010) and rotavirus was used because it is known to be the causative agent in severe gastroenteritis in children, leading to at least 873,000 deaths annually (The Pediatric ROTavirus European CommitTee (PROTECT), 2006; Kapikian *et al*., 2001).

The protozoon *A. castellanii* was selected on the basis that it is a free-living virtually ubiquitous organism which can be isolated from different aquatic environments; secondly, the cyst form of *A. castellanii* is resistant to antibiotics, chlorination, extremes of temperature and biocides. In addition, has been shown to be recoverable and infectious after 24 years of encystment (Marciano-Cabral and Cabral, 2003) and is also reported to internalise other microorganisms e.g. bacteria and viruses (Lau and Ashbolt, 2009; Ly and Müller, 1990; Mattana *et al.*, 2006). Viral survival e.g. echovirus, has been reported to be prolonged following its co-culture with free-living protozoa (Danes and Cerva, 1980).

Importantly, the possibility exists that amoeba-internalised viruses could be protected against adverse environmental conditions and disinfection. This could have important implications for public health, as it may be possible that viruses internalised in protozoa could escape the natural conditions which inactivate viruses in water, or be protected against the artificial treatments used to remove pathogens from water supplies. Furthermore, globally around one billion people have no access to dependable sources of clean drinking water and of mortality resulting from infection by water-borne pathogens, including viruses results in considerable mortality (Black, 1998). A large proportion of the world's population does not have water sterilization facilities and it would be very useful to have access to technology that ensures water is safe. Solar disinfection (SODIS) of contaminated water offers promise as it has shown fast inactivation of microorganisms and in developing countries the low cost and ease of use of solar disinfection of contaminated water makes it a very practical and attractive prospect. Therefore, a further purpose of the current study was to investigate the effect of SODIS on a range of viruses co-cultured with free living protozoa was studied. It was been found that addition of non-toxic additives such as riboflavin could enhance microorganism inactivation by SODIS, and therefore in the current study the potential for enhancement of SODIS by riboflavin was also studied. The study utilises the same viruses to investigate on one hand whether the survival of viruses can be prolonged

when associated with free-living protozoa and also the role that amoeba may play in protecting viruses from SODIS.

The treatment of water contaminated with microorganisms by solar disinfection (SODIS), causes a number of defects in these microorganisms, these include alteration of their plasma membrane that becomes more permeable than normal cells (Bosshard *et al.*, 2010). Thus, SODIS could enhance the internalisation of water-borne human pathogenic viruses in free-living protozoa and provide protection for these viruses from SODIS.

The aims of the study is to investigate the behaviour of viruses and protozoa when they interact in an aquatic environment and on the efficacy of a SODIS to sterilise water contaminated with a number waterborne pathogenic viruses. It was hoped that the information obtained from this study, would add to the body of knowledge of disease agents and their transmission and control. Therefore, help in building towards improved disinfection method and reduction of morbidity and mortality throughout the world.

History and Background

1.1 History

Antony Van Leeuwenhoek (1632-1723) is referred to as the father of protozoology, as he discovered protozoa when he saw them in fresh water in 1674 (Dobell, 1922).
The term Protozoa was derived from two Greek words: 'protos' which means first and 'zoon' which means animals. These microorganisms are single celled eukaryotes belonging to the Kingdom of Protista. Protozoa have been the subject of scientific study for many centuries as, since their first discovery, they have been found in many environments, where they have roles in predation, symbiosis and nutrient recycling, and they can also be the cause of a range of diseases (Marciano-Cabral and Cabral, 2003, Prescott *et al.,* 2002).

1.2 Classification of protozoa

Protozoa were recently reclassified genetically into different phyla (Khan, 2008). The parabasala phylum differs from other phyla by containing only one nucleus and by the absence of mitochondria; an example of this phylum is *Trichomonas*. The second phylum is Cercozoa, a group of amoeba with thread-like pseudopodia, such as *Foraminifera* that lives at the bottom of oceans. Organisms in this phylum vary in size between micrometres and centimetres. In addition, the phylum *Alveolata* has membrane-bound holes called alveoli below its membrane. This phylum is subdivided into three sub-groups: 1) apicomplexans, which include *Plasmodium*; 2) ciliates that include *Tetrahymena*; and 3) dinoflagellates, for example *Gymnodinium*. Radiolaria phylum is another group of amoeba that also has thread-like pseudopodia. The movement of the organisms in this group, by pseudopodia from the central body, is similar to the spokes of a wheel. The Amoebozoa phylum includes free-living amoeba such as *Acanthamoeba* and *Naegleria fowleri*, and parasitic forms such as *Isospora belli*. This phylum has no shell and has round pseudopodia. Another phylum that lacks mitochondria, peroxisomes and Golgi bodies is the Diplomonadida. This phylum has two nuclei with similar size and many flagella; *Giardia* belongs to this phylum. The Euglenozoa phylum is subdivided into two groups. The first group, Euglenids, are photoautotrophic unicellular microorganisms with chloroplasts and flagella. When the

members of Euglenids group reside in the dark, they become chemoheterotrophic phagocytes, for example *Euglena*. The second group, Kinetoplastides, have single large mitochondria. They live in animals, but not all are pathogenic. Both *Trypanosoma* and *Leishmania* belong to the second group. Stramenopila phylum is a complex group that includes protists, both heterotrophic and photosynthetic. Organisms in this group are slime nets, brown algae and water moulds, and are flagellated (Khan, 2008). Finally, the last phylum is Rhodophyta, which includes the red algae; it does not possess either centrioles or flagella (Yoon *et al.,* 2006).

1.3 Free-living protozoa

Protozoa can be either free-living, or parasitic, or both. Parasitic protozoa include *Plasmodium,* the agent of malaria. These have an absolute requirement for a host organism to complete their life-cycle. Free-living protozoa can live without association with other organisms. They have two forms of feeding: by adsorption of dissolved nutrients through the cell membrane or pellicle (saprobic), or by ingesting food particles by endocytosis (halozoic). Halozoic free-living protozoa can ingest bacteria, algae and other protozoa (Jahn and Jahn, 1949). Free-living protozoa can be found in many natural environments, including water, air and soil, and are widespread throughout the world (Prescott *et al*., 2002). Many species of protozoa, belonging to several phyla, are free-living. These include *Balamuthia*, *Naegleria and Sappinia diploidea* (Visvesvara *et al.,* 2007). Amongst the most common are *Acanthamoeba* and *Tetrahymena* (Marciano-Cabral and Cabral, 2003).

The free living protozoa *Tetrahymena* can be found in different aquatic environments. It is a motile organism, using cilia with size range of 50-60 μm for proportion. It belongs to the oligophymenophorea class in the *Alveolata* phylum (Khan, 2008; Sauvant *et al.,* 1999). Four ciliated membranes are found in the oral apparatus of *Tetrahymena*. It is unicellular and does not reproduce sexually because it lacks a micronucleus (Sauvant *et al.,* 1999). Like the other eukaryotes, it contains mitochondria, macronucleus and food vacuoles, and it has a generation time of three to four hours (Larsen *et al.,* 1997; Rachid *et al*., 2008). Since *Tetrahymena* is a unicellular eukaryote, a fast growing organism, it can be cultured in the laboratory in defined media, and has low cost culturing and maintenance. It is used as an animal model in research fields e.g. pharmacological experiments (Gräf *et al.,* 1999; Orias *et al*., 2000).

1.4 Discovery of *Acanthamoeba*

Acanthamoeba was first isolated in 1930 by Castellani, as a eukaryotic cell culture contaminant in *Cryptococcus pararoseus* fungi cultures (Castellani, 1930). In 1930, Douglas placed this amoeba in the genus *Hartmannella* and named it *Hartmannella castellanii* (Visvesvara, 1991). Later, Volkonsky subdivided the *Hartmannella* genus into three genera, when cultured on bacterial agar media, according to their characteristics: 1) *Hartmannella*, which have a smooth-walled cyst; 2) *Glaeseria*, which have nuclear division in the cystic stage; and 3) *Acanthamoeba*, which differ from the others by their double layered cyst, irregular ectocyst and pointed spindles during mitosis (Khan, 2006; Volkonsky, 1931).

1.5 *Acanthamoeba* biology and morphology

The *Acanthamoeba* life cycle has two stages: the active vegetative form, which is the trophozoite, and the dormant stage, which is the cyst. The size of the free-living amoeba trophozoite ranges from 25 to 40 µm. It feeds on yeast, bacteria and algae in the surrounding environment, and it can also feed on nutrients in the liquid environment. Both phagocytosis and pinocytosis are utilised to take up nutrients, and food-cup formation is utilised temporarily to ingest certain food substances e.g. bacteria or yeast (Byers, 1979 and Pettit *et al.* 1996). *Acanthamoeba* is a motile organism which moves slowly using a spiny projections called acanthapodia, which are similar to pseudopodia (Khan, 2006).

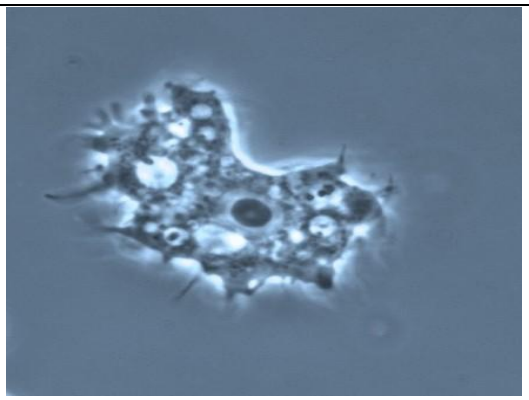


Figure 1.1. The vegetative stage of *Acanthamoeba*; the trophozoite under inverted light microscope. (Courtesy of Dr Simon Kilvington, University of Leicester.)

The acanthapodia, are considered to be a crucial characteristic, allowing them to be differentiated from other free-living protozoa at the genus level [Figure 1.1]. However, identification of *Acanthamoeba* on the species level using morphological characteristics is complex (Daggett *et al.*, 1985; Martinez and Visvesvara, 1997; Stothard *et al.*, 1998).

As with many higher eukaryotic cells, Bowers and Korn (1968), using an electron microscopy, found that the *Acanthamoeba* trophozoite contains digestive vacuoles, Golgi complex, water-expulsion vesicles, mitochondria and both smooth and rough endoplasmic reticula. The cytosol consists of salts, organic molecules and 65% water, and it contains organelles and inclusion bodies which contain nutrients, pigments and secretary products. The cell contents are covered by three layers, called the trilaminar plasma membrane (Khan, 2009). The plasma membrane is composed of approximately 35% protein, 29% lipophosphonoglycan, 13% sterols and 25% phospholipids (Byers, 1979). *Acanthamoeba* reproduces by binary fission and is usually uninucleate, but it has been found to be multinucleate when it is kept in maintenance culture suspension (Byers, 1979; Page, 1967).

1.6 *Acanthamoeba* Biology – the cyst

Acanthamoeba encysts when it comes under unfavourable environmental conditions such as desiccation, changes in pH and temperature and nutrient depletion (Byers *et al.*, 1980; Bowers and Korn, 1969; Chagla and Griffiths, 1974). The *Acanthamoeba* cyst is surrounded by an irregular double wall, separated by a space except at the opercula in the centre of ostioles (from which the trophozoite emerges) [Figure 1.2]. The outer wall (ectocyst) and the inner wall (endocyst) differ from each other in texture. The cyst size ranges in diameter from 13 to 20 µm, and is different between species (Bowers and Korn, 1969; Marciano-Cabral and Cabral, 2003).

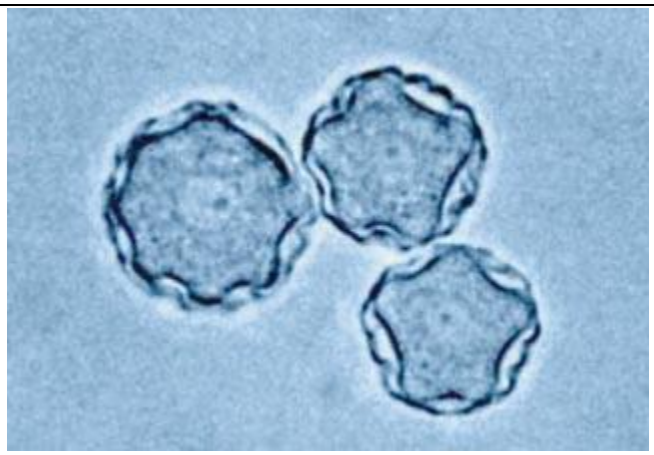


Figure 1.2. The dormant stage of *Acanthamoeba*; the cyst under inverted light microscope. (Qouted from Kilvington, 2000)

Acanthamoeba can be transformed into a cyst, using known cyst inducers (Chagla and Griffiths, 1974; Yang and Villemez, 1994). A monoclonal antibody that binds to a protein on the membrane of *A. castellanii* causes the organism to encyst, and also prevents hatching. Encystment can also be achieved by adding certain inhibitors to the medium, such as 50 mM $MgCl_2$ (Chagla and Griffiths, 1978). Other encystment methods have utilised *Acanthamoeba* cultured in Neff's optimal medium. It was observed that trophozoites started to encyst eight hours after the stationary phase, and >95% encysted at 19 hours post transfer to encystment medium (Stevens and Pachler, 1973; Weisman, 1976).

Acanthamoeba cysts are more resistant to chemicals and disinfectants than the trophozoite stage, and they are also more resistant than *N. fowleri* cysts to chlorination and biocides (De Jonckheere and Van de Voorde**,** 1976; Khunkitti, *et al.,* 1998; Lloyd *et al.,* 2001; Turner *et al.,* 2000). Using samples taken from frozen swimming areas in Norway, it has also been reported that cysts survive at low temperatures (0 to 2°C) (Brown and Cursons, 1977). However, cysts can be killed when autoclaved or treated with either methylene oxide or Freon (Meisler *et al.,* 1985).

As expected, *Acanthamoeba* cysts can reverse back to the trophozoite stage under favourable environmental conditions. For example, cysts were found to be viable for as long as 24 years after cyst formation when they were stored in cold water at 4°C and

hatched on non-nutrient agar (NNA) containing bacteria (Mazur *et al.,* 1995); of 17 isolates of *A. polyphaga* or *A. castellanii,* 14 produced viable trophozoites (Mazur *et al.,* 1995). In order to examine the effect of encystment on virulence, BALB/c mice were given an intranasal challenge of amoebae (trophozoites) which had been encysted for different periods of time. It was found that encysted *Acanthamoeba* isolates stored for 24 years still had a lethal effect on mice, although the effect was less than that produced in mice by encysted *Acanthamoeba* isolates obtained recently (Mazur *et al.,* 1995). This experiment demonstrated that, although *Acanthamoeba* virulence can be sustained for long periods of time through encystment, there may be a gradual loss of potency (Mazur *et al.,* 1995).

1.7 *Acanthamoeba* distribution

Acanthamoeba are almost ubiquitous in the environment; they can be found in water, air and soil (Rivera *et al.*; 1989; Rodriguez-Zaragoza, 1994). They have been found all over the world, and have been isolated from many varied environments, for example air (Kingston and Warhurst, 1969), dust (27%) in 100 samples in Brazil (Teixeira *et al.,* 2009), water, seawater, swimming pools, sewage, air-conditioning systems, tap water, hospital bathrooms, dialysis units (Casemore, 1977), eye washing stations, dental treatment units (Barbeau and Buhler, 2001), contact lenses and lens containers (Seal and Hay**,** 1993; Walochnik *et al.,* 1999). Sawyer (1989) reported that 24 types of amoeba, including five types of *Acanthamoeba* spp., were isolated from soils in Maryland, USA; the farmland soil had been fertilised using municipal sewage waste (Sawyer, 1989). A number of *amoeba* types were present in 79% of 2,454 household water samples collected in the USA; their presence was found in kitchen sprayers (26%) and in shower heads (22%) (Stockman *et al.,* 2010). The authors detected *Acanthamoeba* in higher numbers in biofilm swab samples than in water samples.

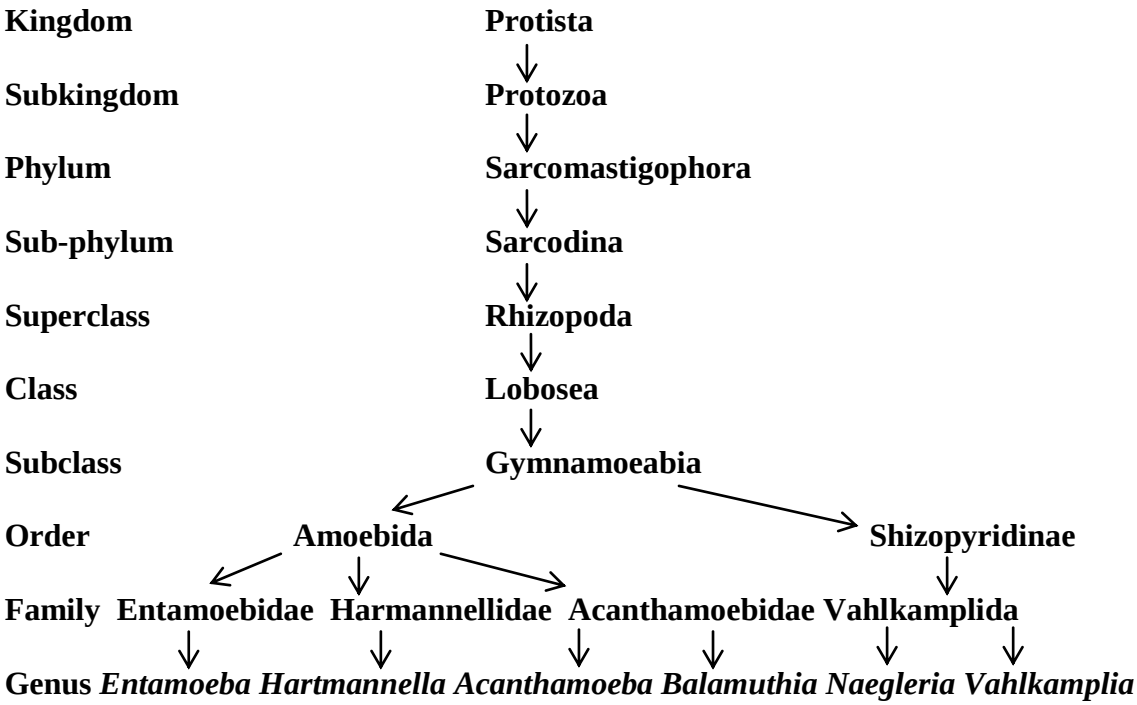
A major reason for the presence of *Acanthamoeba* in tap water was due to inadequate sanitation techniques, or the *Acanthamoeba* entering the domestic water supply system (Bonilla-Lemus *et al.,* 2010). *Acanthamoeba* presence in water is considered a potential health risk. It was reported to be isolated from 30% of tap water in homes in the UK (Kilvington *et al.,* 2004). Pathogenic *Acanthamoeba* can also be found in swimming pools (Caumo *et al.,* 2009). Although the swimming pools contain 1–4 mg/l free

residual chlorine, the *Acanthamoeba* was isolated from 13 (20%) of 65 samples that were collected from swimming pools (Caumo *et al.*, 2009).

Moreover, *Acanthamoeba* have been found as contaminants in mammalian cell, bacterial and yeast cultures (Castellani, 1930; Jahnes *et al.*, 1957; Mergeryan, 1991; Michel *et al.*, 2001) and have also been isolated from animals, including fish and reptiles (Dykova *et al.*, 1999; Sesma and Ramos, 1989). This distribution of *Acanthamoeba* in many different environments makes its contact with other organisms more common.

1.8 *Acanthamoeba* classification

Figure 1.3. Classification of *Acanthamoeba*.
(Quoted from Khan, 2006.)



Acanthamoeba was separated into three morphological groups, based on their cyst shape and size, by Pussard and Pons in 1977 (cited in Khan, 2006) [Figure 1.3]. Group I was selected on the basis that they possess a double walled cyst with a diameter of more than 18µm which has a wide space between the outer and inner cyst walls, and the trophozoites are large. Group I includes four species (*A. tubiashi*, *A. astronyx*is, *A. echinulata* and *A. comandoni*). In group II the cyst size is less than 18 µm and they

differ from the others because the ectocyst (outer layer of the cyst) can be either thin or thick, while the endocyst (inner layer of the cyst) has different shapes: stellate, triangular or circular. Group II contains 11 species (*A. polyphaga, A. hatchetti, A. quina, A. castellanii, A. triangularis, A. divionensis, A. lugdunensis, A. griffini, A. rhysodes, A. paradivionensis* and *A. mauritaniensis*). In group III the cyst size is less than 18µm diameter, with a thin ectocyst and an endocyst with between three to five corners which are gentle. Group III contains five species (*A. royreba, A. lenticulata, A. palestinensis, A. pustulosa and A. culbertsoni*) (Khan, 2006).

However, the classification of *Acanthamoeba* based on cyst morphology is subject to some controversy, as this varies depending on the culture conditions used. In later studies the problem of distinguishing between *Acanthamoeba* strains was investigated using three isoenzyme electrophoresis profiles, and it was suggested that previous standards used for taxonomy were not sufficient to fully determine the taxa at the species level (Daggett *et al.,* 1985; Costas and Griffith, 1985; Stratford and Griffith 1978).

Latterly, new identification methods have been used to distinguish between *Acanthamoeba* species. In one of these methods (randomly amplified polymorphic DNA (RAPD) profiles), the results obtained from Brazilian keratitis isolates did not match with ATCC strains (Alves *et al.*, 2000). Another method was to identify differences in small-subunit ribosomal RNA genes, using DNA amplification to distinguish between pathogenic and non-pathogenic strains of *Acanthamoeba*. However, this method did not provide a decisive tool to differentiate between strains (Howe *et al.,* 1997). Other scientists have used restriction enzyme analysis of *Acanthamoeba* whole-cell DNA to determine the relationship between strains which appear to be identical in their morphology (Kilvington *et al.,* 1991). They found this to be an effective procedure to differentiate between strains. Vodkin *et al.* (1992) used polymerase chain reaction for short ribosomal DNA, but it was only useful in differentiating at the genus level of *Acanthamoeba* and *Naegleria* (Vodkin *et al.,* 1992).

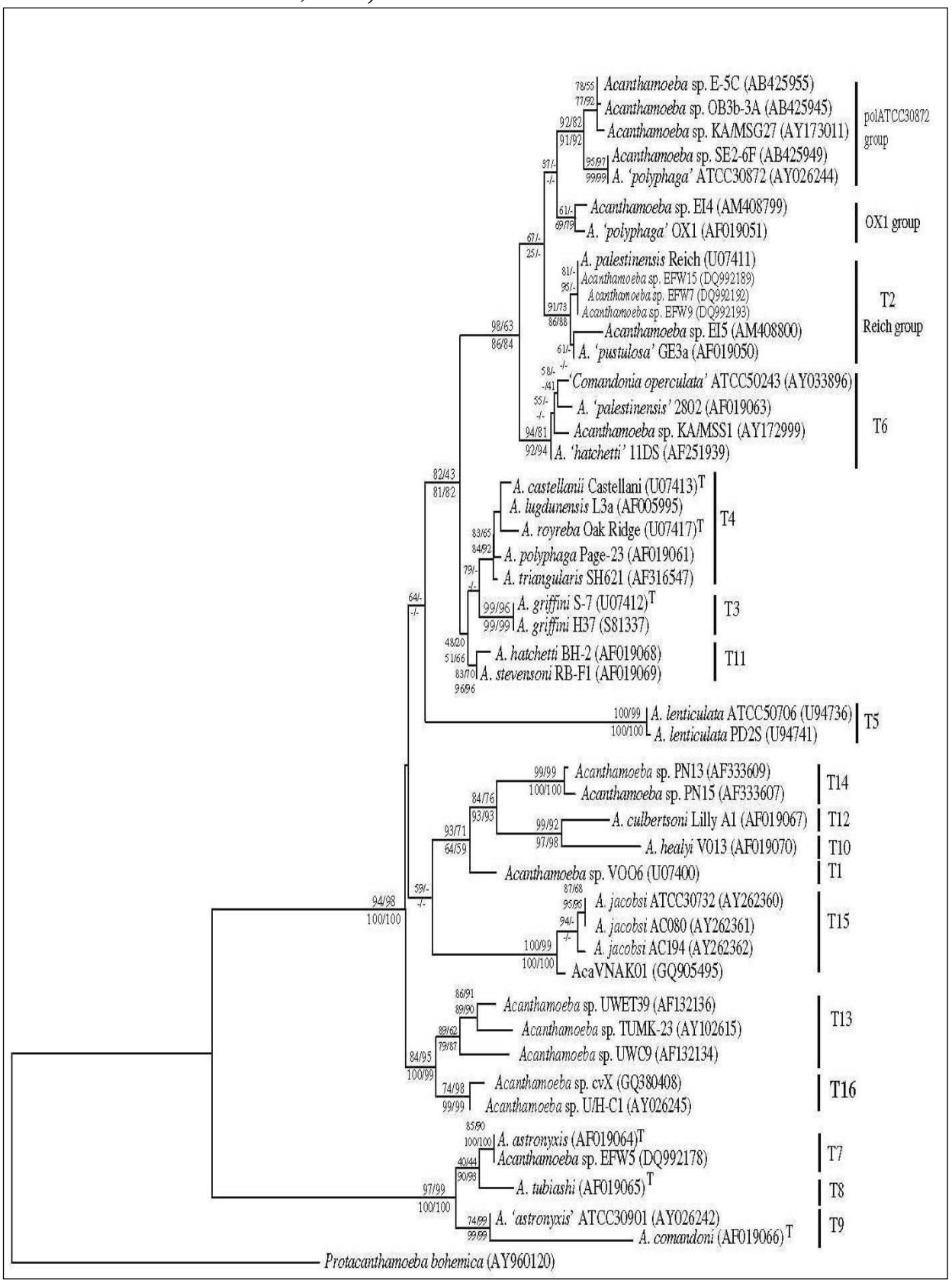
A classification of *Acanthamoeba* strains based on their activity and the profiles of 19 enzymes with 37 strains of *Acanthamoeba* was assessed by Costas and Griffiths using the API Zym method (for enzyme detection) (Costas and Griffiths, 1985). They found only a small correlation between their classification and the cyst morphological

characteristics allocated previously by Pussard and Pons in 1977 (cited in Khan, 2006). Although they used only 19 enzymes, their results suggested that *Acanthamoeba* could be classified into six large groups; some groups include only one strain, while others contain more than three strains. They also mistakenly included *A. hatchetti* in group III, although it is classified in group II, according to Pussard and Pons (1977, cited in Khan, 2006). Kong and Chung used PCR to amplify subunit ribosomal RNA coding DNA (ssu rRNA) (Kong and Chung, 1996). Using RFLP analysis, they found that even in the same genus there were differences between the species. A taxonomic review of the *Acanthamoeba* genus on the basis of sequence comparisons is still in progress (Marciano-Cabral and Cabral, 2003). Although much detail and more *Acanthamoeba* strains were identified and grouped, more improvement is still required for the available identification systems (Kong, 2009).

A study published in 2000 reported that sequence typing of the 18S ribosomal DNA was consistent with the identification based on species morphology (Walochnik *et al.,* 2000). On the basis of analysing the complete sequence of nuclear small ribosomal subunits RNA genes (Rns), Gast *et al.* (1996) reported four different sequence types from 18 strains of *Acanthamoeba*. They identified a number of types based on localised sequence differences in 12 highly polymorphic regions. The first three identified sequence types were found to be specific for a single species. The first sequence type (T1) was from *Acanthamoeba castellanii*; the second sequence type (T2) was from *Acanthamoeba palestinensis*; and the third sequence type (T3) was attributed to *Acanthamoeba griffini*. The fourth sequence type (T4) differed from the previous types as it contained 15 isolates of *Acanthamoeba*, including *A. rhysodes, A. castellanii*, *A. polyphaga* and 10 *Acanthamoeba* keratitis isolates (Gast *et al.*, 1996).

In 1998, Stothard *et al.* extended the earlier classification of the 18S rRNA gene (Rns) when investigating 53 isolates of *Acanthamoeba.* They amplified conserved regions (SSU1 and SSU2) of the 18S rRNA gene, using polymerase chain reaction from the 53 isolates, followed by sequencing and analysing the DNA products. An acceptable taxonomy was made on the basis of these results (T1 - T12). Other scientists like Corsaro and Venditti (2010) added a new genotype group (T16) to *Acanthamoeba* groups classified previously up to T12 by Stothard *et al.,* (1998), by amplifying the 18s rDNA using other primers as can be seen in Figure 1.4 (Corsaro and Venditti, 2010).

Figure 1.4. Phylogenetic tree of *Acanthamoeba* spp.
(Quoted from Corsaro and Venditti, 2010.)



1.9 *Acanthamoeba* growth

Acanthamoeba grows as a monolayer, either on surfaces or in suspension culture using Neff's medium (1% peptone, 1% glucose and inorganic salts) at 30°C (Neff, 1957), with a generation time of six to ten hours. An alternative culture medium described by Adam (1959) consists of 18 amino acids, vitamin B12, thiamine, acetic acid, salts and trace metals, but it has a longer generation time than Neff's medium (53 to 115 hours) (Adam, 1959). A more recent study described a medium composed of three vitamins, glucose, eleven amino acids and six inorganic salts, which gave a mean generation time of ten hours (Shukla *et al.*, 1990).

Acanthamoeba and *Naegleria* can also be grown on a monolayer of mammalian cells such as Hela, HEp-2, Human Embryonic Kidney 293 cells (HEK) and Madin-Darby Bovine Kidney cells (MDBK) (Cursons and Brown, 1978). De Jonckheere (1980) compared pathogenicity, temperature tolerance, cytopathic effect in Vero cells (African Green Monkey kidney cells) and virulence in mice of 36 strains from 19 species of *Acanthamoeba*. The author found that not all pathogenic strains belong to one species, and that both pathogenic and non-pathogenic strains can be found in a single species. Thirty one strains showed a cytopathic effect (CPE) on Vero cells, and although some strains grew at 40°C they were not pathogenic and no relation was found between CPE in cell culture and pathogenicity of *Acanthamoeba* (De Jonckheere, 1980). More recently it has been reported that *Acanthamoeba* was able to destroy a rat nerve cell line (B103 neuroblastoma cells) at both 25°C and 37°C, with the cells being either lysed or ingested by *Acanthamoeba*, as seen under electron microscopy (Pettit *et al*., 1996). *Acanthamoeba* is able to grow on microorganisms or axenic or defined media making it easy to culture and use in laboratory experiments.

Bowers and Olszewski (1983) investigated the ability of *Acanthamoeba* to differentiate between nutritive and non-nutritive particles. Following incubation with an excess of yeast cells, *Acanthamoeba* were allowed a period of rest and then challenged with a further dose of either yeast or plastic particles. Neither of these was taken up by the saturated *Acanthamoeba* cells. However, when given an excess dose of plastic beads and allowed to rest before a further challenge with either plastic beads or yeast, it was found that in the presence of yeast the plastic beads were excreted in order to allow

Acanthamoeba to take up either yeast cells or plastic beads (Bowers and Olszewski, 1983). The authors suggested that *Acanthamoeba* does have some capacity to distinguish between digestible and non- digestible particles.

1.10 *Acanthamoeba* pathogenicity

Acanthamoeba spp. are pathogenic and can cause a sight-threatening disease in healthy humans, *Acanthamoeba* keratitis (AK) (Visvesvara *et al*., 2007). In addition, it causes Granulomatous Amoebic Encephalitis (GAE) in immunocompromised humans that usually leads to the death of the patient (Khan, 2005).

1.10.1 *Acanthamoeba* Keratitis (AK)

Acanthamoeba Keratitis (AK) is a sight threatening disease caused by *Acanthamoeba* spp. which is associated with contact lens wearers, due to the contamination of contact lens containers or the contact lens solutions (De Jonckheere, 1991). AK disease could lead to blindness if not treated (Visvesvara *et al*., 2007). The infection which develops after the attachment of the amoeba to the corneal surface results in an inflammation of the cornea, and both photophobia and ocular pain are common symptoms. The first description of clinical pathogenicity of *Acanthamoeba* was recorded in the UK in 1974 (Nagington *et al.,* 1974). A school teacher presented with inflammation of the cornea, conjunctiva and uvea which led to progressive inflammation over a period of six months. This resulted in the formation of an ulcer in the cornea and blindness which was not resolved with antibiotic treatment. Since then, a number of sub-species of *Acanthamoeba* have been isolated from patients with amoebic keratitis (AK), such as *A. castellanii, A. polyphaga* (Wright *et al*., 1985), *A. hatchetti, A. culbertsoni, A. rhysodes, A. griffini, A. quina, A. lugdunensis* (Bouyer *et al.*, 2007; Ledee *et al.,* 1996; Martinez and Visvesvara, 1997) and *A. triangularis* (Xuan *et al*., 2008). The majority of isolated *Acanthamoeba*-causing keratitis and the majority of *Acanthamoeba* isolated from nature belong to the same morphological group (group II) which both belong to the T4 genotype (Walochnik *et al.,* 2004). Forty cases of AK were recorded at the University

of Illinois, Chicago, between 2003 and 2005 and also other cases have been reported in the UK (Seal, 2003; Joslin *et al.,* 2006).

1.10.2 Granulomatous Amoebic Encephalitis (GAE)

Although AK is perhaps the most common disease associated with *Acanthamoeba* infection, a more severe disease that is usually fatal is Granulomatous Amoebic Encephalitis (GAE) (Khan, 2005). Infection by *Acanthamoeba* occurs via either the skin or the respiratory tract (Khan, 2006). Mannose-binding protein mediates attachment to host cells, and the attachment of *Acanthamoeba* to human cells (e.g. brain microvascular epithelial cells, corneal epithelial cells, neurons) is similar. Following the attachment of *Acanthamoeba* to the host cells, it starts feeding on these cells in the same way that it would feed on other microorganisms (e.g. bacteria). A number of species of *Acanthamoeba* have been isolated from infected patients, for example *A. astronyxis, A. castellanii, A. culbertsoni* (Martínez, 1980), and *A. polyphaga* (Bloch and Schuster, 2005). Infection is generally associated with inflammation of the basal ganglia and cerebellum in the brain (Ma *et al.,* 1990). Pathological analysis has detected both the trophozoites and cyst forms of amoeba in the infected site. The *Acanthamoeba* causing GAE is known to be an opportunistic microbe (Martinez and Jznitschke, 1985), so the disease is generally only found in patients undergoing immune suppression transplantation, or those who have an underlying disease (e.g. HIV/AIDS or bone marrow failure) leading to a down regulation of immunity (Ma *et al.*, 1990; Schuster and Visvesvara, 2004). Treatment of GAE is not standardised and is usually empirical. The antimicrobial agents do not provide satisfactory results for *Acanthamoeba* infection, as *Acanthamoeba* transforms to a cyst with a protective dual-layer cell wall, which has proven resistant to biocides and disinfection. Besides, the diagnosis is usually post mortem (Bloch and Schuster, 2005).

1.11 *Acanthamoeba* interaction with other microorganisms

As *Acanthamoeba* are found in several environments (Storey *et al*., 2004), particularly aquatic environments, they encounter a wide range of other microorganisms, including bacteria, other protozoans and viruses, and they will naturally interact with them. Many

of these interactions involve predation upon other microorganisms, using them as a food source. *Acanthamoeba* was reported to feed on different types of Gram-negative bacteria, including *Klebsiella aerogenes, Escherichia coli, Pseudomonas aeruginosa* and *Alcaligenes faecalis* (Pickup *et al.* 2007; Upadhyay, 1968). Other species of bacteria that are Gram-positive have also been reported as a source of nutrients, e.g. *Arthrobacter simplex, Bacillus megaterium, Bacillus subtilis and Micrococcus luteus* (Weekers *et al.* 1993).

Several microorganisms can, however, survive internalisation within the amoeba and form an association with them. Internalisation of the Gram-negative bacteria *Legionella pneumophila* in *Acanthamoeba* was first studied by Rowbotham (1980). The author indicated that since *L. pneumophila* could be internalised in macrophages, it might be capable of internalisation in *Acanthamoeba* trophozoites. Rowbotham (1980) found that both *A. castellanii* and *A. polyphaga* could internalise *L. pneumophila* inside vacuoles in the trophozoites, where the bacteria subsequently proliferated (Rowbotham, 1980). Subsequently, *L. pneumophila* has been shown to be capable of internalisation and replication in *Acanthamoeba* isolates (Barker and Brown, 1994). The bacteria *L. pneumophila* escapes from digestion inside amoebal vacuoles and replicates in amoeba cytoplasm. The replicated *L. pneumophila* reaches a stage where it fills up the *Acanthamoeba* and causes its lysis and the release of *L. pneumophila* (Barker and Brown, 1994). In addition, it was reported that six species of *Acanthamoeba* can harbour *Legionella* species, e.g. *A. castellanii, A. hatchetti, A. culbertsoni, A. polyphaga, A. palestinensis, A. royreba* (Lau and Ashbolt, 2009).

Association between *Acanthamoeba* and different species of bacteria has also been reported in clinical and environmental samples. The clinical samples were isolated from corneal biopsy, tissue and button samples, and both contact lenses and lens cases (Iovieno *et al.,* 2010). Iovieno and his team found that the majority of *Acanthamoeba* isolates internalise one species of pathogenic bacteria (e.g. *Legionella, or Pseudomonas* or *Chlamydia* or *Mycobacterium*), but one *Acanthamoeba* isolate of was found to comprise two species of bacteria (*Legionella and Chlamydia*). The cytopathic effect on human corneal epithelial cells of *Acanthamoeba* harbouring bacteria was more robust than that of non-infected amoeba (Iovieno *et al.,* 2010). The relationship between both

microorganisms is endosymbiosis. Other species of bacteria were reported to be internalised in *Acanthamoeba*; these strains of bacteria were from the same species (*Shigella dysenteriae or Shigella sonnei*) (Saeed *et al.,* 2009). It was found that these bacteria can be internalised and replicate within *Acanthamoeba* (Saeed *et al.,* 2009). Although both strains were from the same genus of *Shigella*, it was reported that the replication of *S. sonnei* within *Acanthamoeba* is ten times more than when it was cultured alone, while the replication of the second strain, *S. dysenteriae*, within *Acanthamoeba* was found to be 100 times more than when they were cultured alone. The bacterial escape from the *Acanthamoeba* vacuole to the cytoplasm is by lysing the vacuole. The authors indicated that *Acanthamoeba* is beneficial to the bacteria, being a reservoir and facilitating *Shigella* transmission (Saeed *et al.,* 2009).

Other bacteria have also been found to be capable of entering and surviving within free-living protozoa. Ly and Müller (1990) investigated the interaction of *Listeria monocytogenes* when co-cultured with either *Acanthamoeba* or *T. pyriformis*. The results showed *L. monocytogenes* was internalised and decreased in number until day eight, reaching 1 cfu/ml, with a concurrent increase in the number of *Acanthamoeba* (Ly and Müller, 1990). Only 10% of *Acanthamoeba* were encysted after eight days following co-culture with *L. monocytogenes* which started to increase in number. In longer term experiments, at 34 days of co-culture all *Acanthamoeba* had encysted and the *L. monocytogenes* had died. In addition, *L. monocytogenes* was found to be internalised and multiplying in another free-living protozoa, *T. pyriformis;* all the bacterial cells were reported to be ingested in *T. pyriformis* when co-cultured. Unlike *Acanthamoeba* that encysts, the *T. pyriformis* cells were found to be lysed after 8 to15 days post co-culture, releasing viable *L. monocytogenes.* After five weeks of the co-culture all the *T. pyriformis* were dead and consequently the *L. monocytogenes* also died (Ly and Müller, 1990).

In a later study performed by Thom *et al.* (1992), *Vibrio cholerae* was found to survive and multiply inside *Acanthamoeba* and *Naegleria gruberi* when co-cultured in SCGYEM medium (serum-casein-glucose-yeast extract medium), as modified by Aufy *et al.* (1986). They showed that, in the presence of amoebas, the number of *V. cholerae* increased compared to the control. After encystment of both amoebae, the bacteria were recovered from only *N. gruberi* (Aufy *et al.,* 1986; Thom *et al.,* 1992). Another

pathogenic bacterium, Methicillin-resistant *Staphylococcus aureus* (MRSA), has also been reported as having been ingested and replicated inside *A. polyphaga*. Huws *et al*. (2006) explored the interaction between both *A. polyphaga* and MRSA by co-culturing them, at a concentration of 1:100 respectively, in Neff's amoebal saline at 37°C. It was found that MRSA was more virulent and the number increased significantly, with a 1000-fold increase after 24 hours of incubation in the presence of amoeba, compared to the amoeba free control (Huws *et al*., 2006). *Acanthamoeba* was reported to manipulate the mode of bacterial growing environment. An obligate anaerobic bacterium *Mobiluncus curtisii* showed interesting results when co-cultured with *Acanthamoeba* under aerobic conditions; the bacterium was able to grow and replicate within the *Acanthamoeba culbertsoni* (Tomov *et al.*, 1999). Although *M. curtisii* was growing and replicating in *Acanthamoeba* in an aerobic conditions, it neither grew nor replicated when cultured alone in a similar environment. Internalisation and replication of the bacterium within *Acanthamoeba* prolonged for six weeks. *Acanthamoeba* was reported to internalise a number of different species of bacteria (Pagnier *et al.*, 2008). They reported that 86 species of 244 isolated bacteria were capable of growing without digestion in *Acanthamoeba* trophozoites.

Acanthamoeba spp. feeds on microorganisms other than bacteria (Wright *et al*., 1981). Wright and his team stated that *Acanthamoeba* fed on blue-green algae (*Cyanobacteria*), which were taken up by engulfment (Wright *et al*., 1981). Moreover, yeast was reported to be a source of nutrients to *Acanthamoeba*. A receptor on *Acanthamoeba* that recognises and mediates the identification and phagocytosis of *Saccharomyces cerevisiae* was reported. This receptor was suggested to be a mannose receptor (Allen and Dawidowicz, 1990).

It has been shown in several cases that internalised bacteria can be protected from disinfection or harsh external conditions. Many studies reported that *Acanthamoeba* spp. can be a reservoir for different species of bacteria, and these bacteria could benefit from internalisation either by proliferation, by transmission, by protection, or by all of those (Snelling *et al.*, 2006; Thomas *et al.*, 2010). The initial stage of the connection between the bacterium and *Acanthamoeba* must be made prior to ingestion. During the movement of *Pseudomonas fluorescens* and *Pruteus mirabilis* the bacterial flagellum rotates continuously, and once it touches specific sites on *Acanthamoeba* membrane it

holds to it (Preston and King, 1984). Following the attachment of the bacterium, *Acanthamoeba* then phagocytose it. *Mycobacterium avium* was reported to be internalised in *Acanthamoeba* cysts and trophozoites but, unlike other bacteria (e.g. *L. pneumophila*, which is found inside the cyst) *M. avium* was found in the outer cyst wall of *Acanthamoeba* cysts (Steinert *et al.,* 1998). *L. pneumophila* and *M. avium* growing within *Acanthamoeba* were reported to be more resistant to biocides (e.g. rifampin and ciprofloxacin antibiotics) by 1000 times, than when cultured alone (Barker *et al.,* 1995; Steinert *et al.,* 1998).

Two species of free-living protozoa (*A. castellanii* and *T. pyriformis*) were reported by King *et al.,* (1988) to be resistant to chlorine treatment (0.5 to 2.0 mg/l) for 24 hours in their trophozoite stage. The authors extended their study to investigate bacterial resistance to free chlorine, either alone or following co-culture with free-living amoeba. These bacteria included: *Escherichia coli, Klebsiella oxytoca, Klebsiella pneumoniae, Campylobacter jejuni, Legionella gormanii, Shigella sonnei, Salmonella typhimurium,* and *Yersinia enterocolitica* (King *et al.,* 1988). All the bacteria species tested were internalised in each of *A. castellanii* and *T. pyriformis*, and enhanced the bacterial resistance to disinfection with chlorine when co-cultured together. The authors also reported that pathogenic bacteria, when internalised in *T. pyriformis*, were resistant to free chlorine by over 50 times more than when cultured axenically.

Acanthamoeba were found to be resistant to treatment with 128 ppm of sodium hypochlorite (NaOCl) disinfectant at either 22 or 46 hours (García *et al*., 2007). The authors also reported that the viability of amoeba when treated with a higher concentration of NaOCl (256 ppm) was reduced by over 80%. In addition, *Acanthamoeba* treatment with 256 ppm NaOCl was carried out when infected with different strains of *Legionella*. The reduction of amoeba viability was extremely marked, with only 7-40% destroyed. A 50% reduction was achieved after infected *Acanthamoeba* was treated with 512 ppm of NaOCl (García *et al*., 2007).

In addition, acidic environmental tolerance for internalised *C. jejuni* within *Acanthamoeba* was investigated following co-culture (Axelsson-Olsson *et al. 2010*). The *Acanthamoeba* were reported to enhance the tolerance of *C. jejuni* to an acidic environment (pH 4-5) for as long as 20 hours, and when acidity was increased (pH 2),

the bacteria was able to withstand it for five hours. It was found that following co-culture of *C. jejuni* with *A. polyphaga* trophozoites, *C. jejuni* acquired resistance and was more viable than that *C. jejuni* alone. The acidic environment increased the association between *C. jejuni* and *A. polyphaga*, either by attachment or by internalisation. It was concluded that *A. polyphaga* could provide protection to *C. jejuni* from extreme conditions (Axelsson-Olsson *et al. 2010*).

Baré and his team (2010) investigated the interaction between *A. castellanii* and five different strains of *C. jejuni*. The authors found that C. *jejuni* was able to be internalised and replicate within *A. castellanii* and that *Acanthamoeba* prolongs the survival duration of five strains of *C. jejuni* (Baré *et al.,* 2010). They also highlighted that environmental conditions affect the interaction between the organisms; the viability of *Acanthamoeba* falls significantly when co-cultured with *C. jejuni* at 37°C in an anaerobic environment. The authors suggested that *C. jejuni* escaped from *Acanthamoeba* digestion by some means other than the usual mechanism; they suggested that the internalised bacteria were contained within bacteriosomes. Another well-known bacterium that is pathogenic to humans and usually causes ulcers in their digestive system is *Helicobacter pylori* (Winiecka-Krusnell *et al.,* 2002). It was found to survive within *A. castellanii* vacuoles for many weeks. It also showed 100-fold increase after one week of co-culture with living *A. castellanii*. It was suggested that this interaction between these organisms would facilitate the transmission and spread of *H. pylori* (Winiecka-Krusnell *et al.,* 2002).

In addition, *Acanthamoeba* cysts were reported to be resistant to 100 mg/l of chlorine and to withstand very high temperatures up to 80°C (Storey *et al.,* 2004). Following the heat and chlorine tolerance experiment of *Acanthamoeba* alone, Storey *et al.* assessed the tolerance of six different strains of *Legionella* to either chlorine treatment or thermal environment when each of the strains was co-cultured with *Acanthamoeba* in different flasks. Their results showed increased resistance of *Legionella* to thermal conditions reaching 80°C, following their co-culture with *Acanthamoeba*. Despite, *Acanthamoeba* providing the bacterium with protection from thermal conditions, the *Legionella* showed more susceptibility to chlorine than before co-culture with the amoebae. Although *Acanthamoeba* internalise bacteria, not all the species within the same genus or the

strains that belong to the same species act similarly. *Burkholderia cepacia complex* was found to be internalised and replicate within *Acanthamoeba*, while other species (e.g. *B. vietnamiensis strain* CEP040 and *B. cenocepacia H111*) do not replicate (Lamothe, *et al.,* 2004; Marolda *et al.,* 1999). The *B. cepacia complex* was found to be protected from antibiotic treatment within the amoebal cyst (Marolda *et al.,* 1999), and similarly *Vibrio mimicus* within *A. castellanii* (Abd *et al.,* 2010). In addition, Gentamicin is usually utilised to inactivate bacteria outside *Acanthamoeba* cysts when co-cultured, as a confirmation of internalisation (Saeed *et al.,* 2009). The authors reported that *Shigella* were still viable and protected within *Acanthamoeba* cysts following the treatment of the co-culture suspension with Gentamicin antibiotic.

As mentioned earlier, *Acanthamoeba* can be found in different environmental and man-made environments, and even in clinical samples; it was found that pathogenic microorganisms share these environments with *Acanthamoeba*. Both *Acanthamoeba* and *Legionella* were present, at 8.8% and 14.7% respectively, in spring water from 34 sites in Taiwan (Hsu *et al.,* 2009). More *Legionella* were detected within *Acanthamoeba* that was isolated from the water samples. In addition, water treatment plants showed the presence of other bacteria and *Acanthamoeba*. The detection results showed that, in 125 samples collected from the treatment plants in Spain between August 2006 and July 2007, *Legionella* was the highest detected organism (in about 42% of the samples), followed by *Acanthamoeba* (in over 27%), then *Mycobacteria* (in 21.5%) and that the least prevalent one was *Chlamydia* (in 11%) (Corsaro *et al.,* 2010).

The feeding of *Acanthamoeba* on other microorganisms, and the internalisation of bacteria that escape from digestion, replicate and become more virulent, is a critical point. *Acanthamoeba* could make other microorganisms resistant to different biocides or disinfectants. In addition *Acanthamoeba* could become a vehicle for pathogenic microorganisms, subsequently spreading more diseases worldwide.

1.12 Protozoa and their interaction with viruses

Although not extensively studied, associations between amoeba and viruses may also occur in nature. Virus-like particles (VLP) in protozoa were reported using electron microscopy, and VLPs were observed in *Plasmodia, Naegleria, Leishmania* and

Entamoeba (Wang and Wang, 1991). VLPs were only occasionally observed, except in *Entamoeba*, and could not be differentiated from prokaryotic inclusions within the eukaryotic cells. Three types of VLPs were found in *Entamoeba*, and these were classified based on their size and location within host cells. Those ranging from 75-80 nm were only found in the cytoplasm, while other VLPs of 7 nm and 17 nm were located primarily in the nucleus. *Trichomonas vagainalis* virus (TVV), which is a 5.5 kb dsRNA virus, was the first identified and purified dsRNA virus from a protozoan (Wang and Wang, 1991). Later, Wang *et al.* (1993) found that *Giardia lamblia* Virus (GLV) nucleic acid is also a linear dsRNA like TVV nucleic acid, and that *G. lamblia* can harbour as many as 5×10^5 GLV per cell without affecting the rate of growth. The genome of GLV was sequenced and found to be 6,100 nucleotides in length (Wang *et. al.*, 1993).

Mimivirus is a giant virus associated with *Acanthamoeba polyphaga.* It was first isolated in 1992 from a water cooling tower in Bradford, England, and was identified in 2003 (La Scola *et al.*, 2003). The virus is 400-750 nm in diameter and is surrounded by an icosahedral capsid. It has double-stranded DNA, with a circular genome of 1.2 Mb (Claverie *et al.*, 2009; Raoult *et al.*, 2004). The entry of mimivirus into *Acanthamoeba* was investigated, using electron tomography and cryo-scanning electron microscopy, by Zauberman *et al.* (2008). They first cultured *Acanthamoeba polyphaga* and then co-cultured them with mimivirus. Infected cells were examined under an electron microscope after treatment. They found that the genome delivery of the virus to its host involved opening up five icosahedral faces in the capsid. This opening at the unique vertex of the capsid was termed the "stargate", and it allows the viral DNA to pass through into the cell. After replication of viral DNA inside *Acanthamoeba*, it is packaged through a face-located opening, and the entry portal was then closed at the end of the packaging process (Zauberman *et al.*, 2008). In the same year La Scola and his team (2008) found a new virus associated with mimivirus, after *A. castellanii* was infected with mimivirus. They reported that the virus 'sputnick' size is 50 nm, and part of its circular dsDNA (18.343 kbps) is related to other viruses that infects other microorganisms like bacteria, Eukarya and Archaea. It is a virus inside a larger virus, itself inside *Acanthamoeba*. This smaller virus was termed a 'virophage', and it was suggested that it could be a vehicle for transporting genes between giant viruses (La

Scola *et al.*, 2008). This internalisation of a mimivirus indicates the ability of *Acanthamoeba* to internalise large and/or DNA viruses.

Only a few studies have been performed to investigate any interaction of viruses with protozoa. The survival of two enteric virus types, poliovirus and echovirus, when co-cultured with *A. castellanii* was studied by Danes and Cerva (1981). Poliovirus 1, 2 and 3 and echovirus 4 and 30 were used in the co-culture with *A. castellanii* Neff's strain. The co-culture was established in two different media: a 2% Bacto-Castine solution in distilled water and a 2% Bacto-Castine solution in waste-water. The experimental period was up to 21 days and the co-culture was incubated in a stationary incubator at 37°C. The results indicated that no internalization of viruses occurred within amoeba (Danes and Cerva, 1981). After three years, the same authors investigated the presence of polio (type 1 and 2) and echovirus 30 in association with *Tetrahymena pyriformis* amoeba in sewage sediment. Although the authors did not assess viral internalisation of viruses in amoeba, they suggested that enterovirus presence with *T. pyriformis* served to prolong enterovirus contamination of municipal sewage water (Danes and Cerva, 1984). These studies indicate the difficulty of internalising picornaviruses in free living protozoa in the laboratory.

In another study, a different enteric virus, coxsackievirus B3, was reported to be adsorbed onto the surface of *A. castellanii* cells and then to accumulate inside the cells following co-culture (Mattana *et al.*, 2006). It was found that after encysting *A. castellanii* for six months, followed by hatching of the cysts, infectious viral particles were released (Mattana *et al.*, 2006). In addition, only one study has reported finding an association between an enteric virus and an amoeba in nature. Lorenzo-Morales *et al.* (2007) isolated *Acanthamoeba* strains from tap water in the Canary Islands and surveyed them for the presence of human adenoviruses, in order to investigate a potential reason for observed co-infections with these agents. The authors detected various adenovirus types and suggested that *Acanthamoeba* could act as a potential reservoir of adenoviruses in the environment and be a vehicle for their transmission. These studies show the possibility in strains of *Acanthamoeba* for the internalisation of water-borne pathogenic viruses, in nature or experimentally, and for it to become as a

transporter. This could increase the spread of more diseases, including those caused by water-borne pathogenic viruses, especially in their shared environments, e.g. water.

Protozoa were shown to internalise pathogenic viruses indirectly by engulfing already infected mammalian cells (Pindak *et al.,* 1989). Herpes simplex virus and reoviruses (HSV) were reported to be internalised in *T. vaginalis* following their co-culture with mammalian cells that were previously infected with these viruses (Pindak *et al.,* 1989). The authors were able to detect viruses for 6 and 9 days following co-culture. A more recent study reported that more virulent viruses, human immunodeficiency viruses (HIV-1), were internalised in *T. vaginalis* following its co-culture with T-lymphocytic cells that were already infected with these viruses (Rendon-Maldonado *et al.,* 2003).

Tetrahymena thermophile was reported to internalise and inactivate MS2 bacteriophage (Pinheiro *et al.,* 2008). After the co-culture of *T. thermophile* with high number of MS2 (10^{10}), the authors indicated that the virus was visualised in food vesicles using fluorescent microscope and inactivated more by viable cells when compared with bacteriophage control. They suggested that the inactivation of the bacteriophage is by digestion following engulfment.

1.13 Viruses in the aquatic environment

It was found and reported that many human pathogenic virus types can be found in aquatic environments (Wyn-Jones and Sellwood, 2001). The consumption of or interaction with water contaminated with viruses would potentially expose humans to infection, with a probability between 10 and 10,000 higher than for other pathogenic microorganisms like bacteria (Haas *et al.,* 1993). The presence of these viruses in water environments is one of the potential health risks for both humans and animals who share the same environment. Concern is increasing about water-borne pathogenic viruses that are probable contamination sources, especially enteroviruses. For example, coxsackievirus group B serotypes 1-6 have been detected in coastal bathing water in Northern Ireland, with the most prevalence serotypes being B3, B4 and B5 (Hughes *et al.,* 1992).

Räsänen *et al.* (2010) found that rotavirus was the most common virus of all the investigated microbes in sewage water following gastroenteritis outbreak in a

community in Finland (Räsänen *et al.*, 2010). The detection of enteroviruses RNA or DNA also showed their presence in the seawater; both adenovirus and coliphage were present (33% and 42% respectively) in 12 collected samples in Southern California, USA (Jiang *et al.*, 2001). Kittigul *et al.*, (2005) reported that rotavirus was present in 20% of ten rivers, 27% of 30 canals and 25% of 40 sewage samples at the time of their investigation in Bangkok, Thailand (Kittigul *et al.*, 2005). Rotavirus, which is responsible for diarrhoea in children, has been found in high percentages in both raw and treated sewage water in many countries, including developed countries (Gerba, *et al.* 1996). Rotavirus was also reported to be the most common pathogen causing diarrhoea in an outbreak in Gourdon, France in 2000. Rotavirus group A was found in 71% of 24 samples, followed by presence of *Campylobacter coli* in 32% of 35 samples and norovirus was detected in 21% of 24 faecal samples. The authors suggested that infections originated from a groundwater source due to a malfunction of the chlorination system (Gallay *et al.*, 2006).

Outbreaks of meningitis, encephalitis and myocardial disorders were reported in Minsk, Belarus. Three enteric viruses were detected as the agents of the outbreak: echovirus 30 and 6, and coxsackievirus 5 (Amvrosieva *et al.*, 2006). Following investigation, the viral source was found to be mainly tap water, but also other water sources (bottled water and open reservoirs) in the towns (Amvrosieva *et al.*, 2006). The most common enteric viruses that cause meningitis in children are the coxsackieviruses within the B group (Berlin *et al.*, 1993; Zhong *et al.*, 2009). Another enteric virus that caused a serious outbreak was the poliovirus; it was reported to be the causative agent of acute paralysis in 21 patients in Hispaniola in 2000. All three types (1, 2 and 3) of poliovirus were positive in 23 out of 55 (41.8%) river and sewage water samples (Vinjé *et al.*, 2004). More than one type of enteric virus can be a source of a single outbreak. Maunula *et al.*, (2009) reported that five enteric viruses were detected following gastroenteritis outbreak in Nokia, Finland in 2007. The outbreak was responsible for the infection of 300 people with enteric viruses. There was a correlation between the drinking water and the outbreak; a leak of treated sewage water was the source of the viruses in the drinking water system. The authors found that in majority, the causative agents of this outbreak were attributed to enteric viruses (present in 80% of 300 samples); enterovirus (3.7%), rotavirus (7.5%), adenoviruses (18.2%), astrovirus (19.7%) and norovirus (30%) (Maunula *et al.*, 2009).

He *et al.* (2009) reported that rotavirus was present in raw water, treated water and tap water samples from Beijing, China. The presence of the virus was highest in tap water. Rotavirus group A was detected in all the collected water samples, 22.4% in 32 samples of tap water, while the treated water had 12% in 77 samples and 26 samples or raw water contained 35% positive samples. A study was performed by Lee and Kim (2002) to investigate the presence of viruses in tap water in urban areas in south Korea. The authors reported that enteroviruses 48% and adenoviruses 39% were present in 11 tap water samples. The authors indicated that the annual risk of infection by adenoviruses is 8.3/10000, which is higher than the recommended ratio set by the US Environmental Protection Agency (EPA), which recommends that the probability of infection should not exceed 1:10000 $yr^{-}1$ (1 infected patient per 10,000 of the population per year) (Lee and Kim, 2002).

In addition, Lee *et al.* (2008) reported the presence of coxsackieviruses and reovirus following an investigation of surface waters (lake and river). The presence of coxsackievirus and reovirus was similar in total: 43% and 42% in 21 samples collected from lakes and rivers respectively in Republic of Korea (Lee *et al.*, 2008). Craun *et al.*, (2006) indicated following previous studies of water-borne outbreaks since 1971 until 2006, that there were some limitation of the investigation of these outbreaks and that not all water-borne outbreaks were documented and not all water-borne outbreaks were explored and acknowledged (Cliver *et al.*, 2006). In addition, norovirus, which is a common etiological agent of non-bacterial gastroenteritis in humans, was reported in a number of outbreaks caused by water-borne viruses (Maunula, 2009; Kvitsand and Fiksdal, 2010).

Other enteric viruses, e.g. Norwalk virus, were reported to be the source of an outbreak of waterborne Norwalk virus gastroenteritis in a resort in Arizona, USA. The outbreak, which was reported in 900 people, was due to faecal coliforms and Norwalk virus. After investigation it was later discovered that it was caused by a minute leakage from a sewage treatment source to the resort's well (Lawson *et al.*, 1991). In addition, an outbreak in Finland reported that around 25-50% of the population in a town called Finish had gastroenteritis caused by a number of viruses, such as Norwalk, rotavirus (A and C) and small rounded virus (SRV). This outbreak was due to a spring flood that

contaminated the drinking water (Kukkula *et al.,* 1997). An outbreak of hepatitis in a north Georgia trailer park with a shared well was found to be due to the presence of hepatitis A virus antigen, and the virus was still detectable by enzyme-linked immunoassay 28 days after the onset of the outbreak (Bloch *et al.,* 1990).

Another hidden potential hazard is the community of either mixed microorganisms or of one species (e.g. *Pseudomonas aeruginosa*) that are attached to a surface which is termed 'Biofilm' (O'Toole *et al*., 2000). A range of microorganisms were reported in biofilms (e.g. bacteria, fungi, viruses, bactriophages and protozoa) and when released into drinking water they can cause water-borne diseases (Helmi *et al.,* 2008; Langmark *et al*., 2005; O'Toole *et al.,* 2000; Skraber *et al*., 2005). The microorganisms found in biofilms were reported to be resistant to antibiotics (Gilbert and Foley, 1997). A pilot scale study was performed by Helmi *et al.* (2008) to detect the survival time of protozoa and virus in biofilm. They reported that infectious viruses and viable protozoa attached to the biofilm following one hour of inoculation. Infectious viruses were detected up to six days later, and at 34 days it was possible to detect viable protozoa and viral RNA by RT-PCR (Helmi *et al*., 2008).

Although enteroviruses can be detected in waste-water at certain times (e.g. enterovirus in June, and norovirus from January to March), they are present for longer periods at a constant concentration in biofilms, and could be present all year (Skraber *et al.,* 2007; Skraber *et al.,* 2009). Their presence for longer periods makes them potentially more hazardous, as diseases could spread at different times than their epidemical seasons. The biofilm contains other viruses that are not detected in waste-water, which means that it preserves these viruses for long time. These naturally occurring biofilms, which are considered as small shared environments, include protozoa, bacteria and viruses (Skraber *et al.,* 2007). The pathogenic viruses and other microorganisms in biofilms were reported still to be present even with the presence and usage of disinfection reagents and methods (Storey and Ashbolt, 2001).

It is also important to mention that theses microbes within biofilms are protected from either biocides or dryness, and that they start a new infectious cycle when released (Lacroix-Gueu *et al.,* 2005). The presence of infectious viral particles is prolonged in

the presence of clay particles, within the biofilms, which increases the chances of infection following its detachment (Lacroix-Gueu *et al.*, 2005; Quignon *et al.*, 1997).

The presence of pathogenic viruses in biofilms is not problematic in itself, but the main concern is the release of these viruses into water distribution systems. When the pathogenic viruses are released into drinking water, they become disease causative agents to both humans and animals (Storey and Ashbolt, 2001). Since many viruses were found in environments that previously showed the presence of *Acanthamoeba*, this mutual environment will increase the probability of interaction between them. This could lead to internalisation and protection of water-borne pathogenic viruses within *Acanthamoeba*. Waste-water gathers human discharges that contain both mammalian cells which slough of in faeces (Alexander, *et al.*, 1998) and enteric viruses that shed in the same environment (Zhang and Farahbakhsh, 2007, Okoh *et al.*, 2010). It has been reported that around 120 detected viruses in waste-water are pathogenic.
Since water was found to be a mutual environment for many microorganisms (e.g. water-borne pathogenic viruses, bacteria and free living protozoa and mammalian cells) there is an increase in the probability of interaction between these microorganisms that might lead to resistance to disinfectants.

1.14 Viruses

Viruses are distinct from other microorganisms in the environment as they are smaller than bacteria. The nucleic acid is composed of small segment(s) and it is covered with a protein coat. Likewise, they are unable to metabolise independently of host cells, which are induced to facilitate viral replication (Condit, 2001). Viruses are classified and grouped according to their genome type and structure: single stranded DNA (ssDNA), double stranded DNA (dsDNA), positive-sense single stranded RNA (+ssRNA), negative-sense single stranded RNA (-ssRNA) and double stranded RNA (dsRNA) as an informal classification. Moreover, viruses are classified universally following the hierarchy levels or orders, family, subfamily, genus and species (Fauquet *et al.*, 2005).

1.14.1 Coxsackievirus

Coxsackievirus (Figure 1.5) was discovered in 1948 by Dalldorf and Sickles when they were working on poliovirus, and was named after the town where they discovered it (Coxsackie, New York) (Dalldorf, 1948). It belongs to the *enterovirus* genera and the family *picornaviridae*. It is a non-enveloped virus with a linear positive-sense single stranded RNA genome (+ssRNA) enclosed in an icosahedral protein capsid. Four structural proteins make up the viral capsid: VP4 on the inner surface shell, and VP1, VP2, VP3 on the outer surface. The virus is 30 nm in diameter, acid stable and remains infective even below pH 3.0. There are two groups: group A has 24 types and group B six types, as defined by serological classification (Gifford and Dalldorf, 1951; Pallansch and Roos, 2001). Pathogenically, group A coxsackieviruses have been shown to infect heart muscles and to cause severe inflammation of the tonsils and posterior pharynx, meningitis, poliomolytis, epidemic myalgia, diarrhoea and fever (Pallansch and Roos, 2001; Top, 1952). Coxsackie A16 is the cause of hand, foot and mouth disease, which affects humans (Hosoya *et al.,* 2007). Group B coxsackieviruses tend to infect the central nervous system, the exocrine pancreas and the liver, and to cause meningitis, summer grippe, minor febrile illness, chest and abdominal pain, and mild paralytic disease with residual atrophy (Zaoutis and Klein, 1998). Coxsackievirus B3 has been shown to be involved with myocarditis, and coxsackievirus B1-5 to cause limb paralysis (Yui and Gledhill, 1991), meningoencephalitis, hepatitis and myocarditis (Chapman *et al.,* 1994; 1997; Hyypiä *et al.,* 1993). Coxsackievirus B5 was first cultured from the cerebrospinal fluid of a 28 year old woman in the UK who died after four months in a coma resulting from severe necrotizing encephalitis (Heathfield *et al.*, 1967). It has also been isolated from children with diarrhoea from an outbreak in Australia (Ferson and Young, 1992). The coxsackievirus receptors are ICAM-1 (intercellular adhesion molecule 1) or CD54, DAF (decay accelerating factor) or CD55, Car (coxsackievirus-adenovirus receptor) or the integrin $\alpha_v\beta_3$. The route of infection is faecal-oral, although respiratory transmission has also been reported (Bopegamage *et al.,* 2005, Shafren *et al.,* 1997; Xiao *et al.,* 2001).

Reverse transcriptase polymerase chain reaction was used to evaluate the epidemiology and typing of human enteroviruses as etiologic agents of meningitis in Cyprus by Richter *et al.* (2006). They found that the most prevalent enteroviruses were echovirus

50 (55.5%), echovirus 13 (15.1%), echovirus 6 (13.8%) and echovirus 9 (8.3%), during their study from 2000 to 2002. While other viruses were found to be rare in the collected samples and these viruses include coxsackievirus B2, echovirus 4, enterovirus 71, coxsackievirus B1, B5 and A6in 218 patient samples. From this study they concluded that the serotype distribution of enteroviruses matched that found in trans-European based studies, and they suggested that tourism may play a significant role in viral spread (Richter *et al.*, 2006).

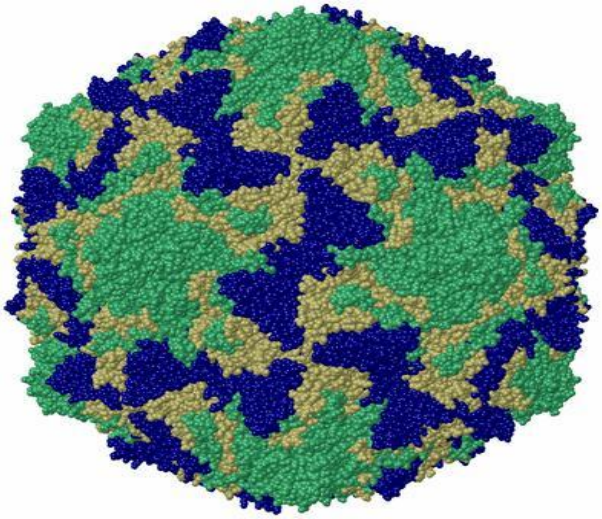


Figure: 1.5: Coxsackievirus B3.
(Quoted from; Jena Library of biological macromolecules).

1.14.2 Poliovirus

Poliovirus belongs to the family *picornaviridae; it* was discovered by Landsteiner and Popper in 1908 (cited in Peters, 2004), and they were able to show that it was the causative agent of polio. They first tried to culture a filtrate of serum from a nine year old boy who had died from polio without culturing any bacterial growth. Injecting the filtrate into rabbits and guinea pigs was unsuccessful, but they later tested monkeys, which are closer to humans. The symptoms started to show on the monkeys within ten days. Poliovirus has three serotypes, P1, P2 and P3, which all cause paralysis (Morgan, 1949; Peters, 2004; Racaniello, 2006). The virus contains 60 copies of the capsid proteins VP1, VP2, VP3 and VP4, and one copy of the positive single stranded RNA (+ssRNA) (He *et al.,* 2000). All three types share the same receptor (CD155) which is also termed the poliovirus receptor (PVR) (Mendelsohn *et al.,* 1989; Racaniello, 2006). In addition, a study was performed in Kuwait by Olive *et al.,* (1990) to detect and differentiate between picornaviruses in throat washes, nasal swabs, stools and cerebrospinal fluid (CSF) taken from patients suffering from respiratory infections. They found that the detection sensitivity of viruses using RT-PCR was high, showing positivity with as low as one plaque forming unit (p.f.u.) per sample tested on serially diluted CSF and stool samples known to be positive for poliovirus. They also differentiated between viral strains such as wild-type rhinoviruses, polioviruses (1, 2 and 3), coxsackieviruses A (1, 9, 16, 21 and 24) and B (1, 2, 3 and 5), coronovirus (229E) and echoviruses (Olive *et al.,* 1990).

1.14.3 Picornaviruses replication cycle

The total replication cycle of poliovirus (Figure 1.6) occurs in the cell cytoplasm. Following the attachment of a virion to its cell surface receptor (e.g. PVR for poliovirus), it enters the cell cytoplasm by endocytosis. A polyprotein which is decoded by the viral +ssRNA is a precursor for other proteins for the viral replication. Then, three proteins (P1, P2 and P3) are produced upon the cleavage of the precursor protein by internal proteases. P1 is then divided into three proteins (VP1, VP2 and VP3) that contribute to the viral capsid assembly. Non-structural proteins, replicase and VPg (which is known as the primer for the virus replication in combination with certain residues) are produced following cleavage of both P2 and P3. These proteins are

responsible for the modifications of the host cell that ends by the lysis host cell. Further mRNA that lacks VPg is produced when replicase interferes with replicative intermediates (RI-1 and RI-2). Then a replication compartment is formed by the association of cell membrane with the replication substances. Replication stops after the replication process has formed enough capsid protein and translation of the newly +ssRNA is synthesised repeatedly. The virion capsid becomes mature when the newly produced +ssRNA with VPg at its 5' end enters the procapsid. Finally, virions are released following cell lysis (Wagner *et al.,* 2004).

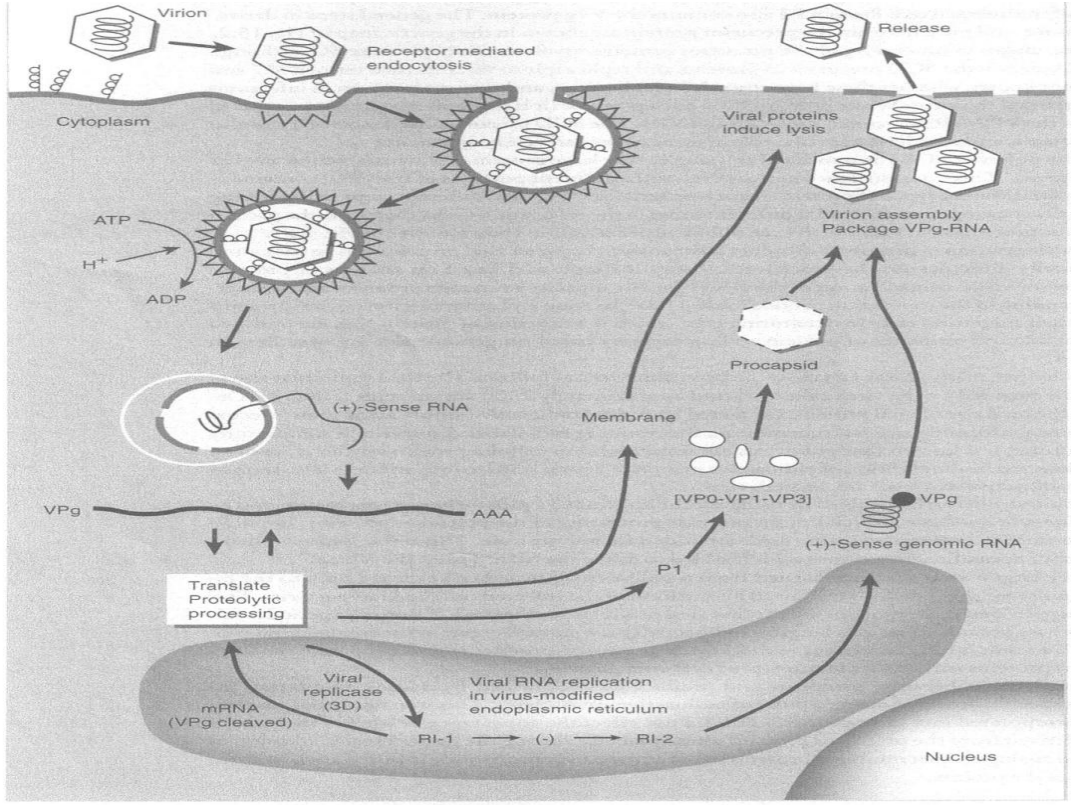


Figure 1.6. The replication cycle of poliovirus. (Quoted from Wagner *et al.,* 2004, p. 238.)

1.14.4 Rotavirus

Rotavirus was first discovered in Australia in 1973 by Bishop (cited in Flewett and Woode, 1978), who used electron microscopy of duodenal mucosa from children with acute gastroenteritis not caused by bacteria. It was first called orbivirus, but then later "rotavirus" was suggested by Flewett and Woode; the name derives from the Latin "*Rota*", because it resembles a wheel (Flewett *et al.* 1974; Flewett and Woode, 1978). Rotavirus belongs to the Reoviridae family and is classified into seven types: A, B, C (which infect both humans and animals) and D, E, F and G (which infect only animals).

In each group there are a number of serotypes classified on the basis of their reactivity in plaque reduction neutralisation, which is determined by the outer proteins VP7 and VP4. Fourteen serotypes of VP7 and 20 of VP4 were reported in group A rotaviruses. It is a non-enveloped virus with a double stranded RNA (dsRNA) genome. Each of the 11 segments within the genome code for one protein or more. It has a size of about 75-100 nm, and the viral particle is covered with an icosahedral protein capsid that is composed of three layers: an outer layer, an intermediate layer and an inner layer. From the outer layer, 120 spikes of viral protein 4 (VP4) extend outside the viral capsid. The capsid also contains an enzyme for RNA replication, in addition to other enzymes (Kapikian *et al.*, 2001; Estes, 2001).

The most common infectious type of rotavirus is group A, and it is estimated to be responsible for at least 873,000 deaths per year of children under five years old in developing countries, while in developed countries like the USA it is estimated to be the agent of diarrhoea disease in about 2.7 million cases per year (Kapikian, *et al*. 2001). In addition, it has been estimated that between 72,000 and 77,000 children per year require hospitalization in European countries due to infection with the virus (The Pediatric ROTavirus European CommitTee (PROTECT), 2006). The virus infects the villous epithelial cells of the small intestine, causing mitochondrial swelling and shortening of the villi. The virus can be detected using commercially available kits, such as serological tests which detect the antigen by latex agglutination and ELISA. Other detection methods are laboratory based techniques such as electron microscopy (which detects viral particles) and polymerase chain reaction (which provides great sensitivity by amplifying viral RNA) (Kapikian *et al.*, 2001; Mebus *et al.*, 1977; Koopman and Monto, 1989; Sanekata *et al.*, 1981). The rotavirus pathogenicity extends the general symptoms (e.g. diarrhoea, fever and vomiting) by the detachment or sloughing of epithelial cells from the small intestine (Horino *et al*., 1985; Lynch *et al*., 2003). These excreted epithelial cells contain rotaviruses that are transmitted in the sewage system in the populated area. As a result, the sewage water system becomes inhabited by these viruses which could interact with other microorganisms or with the water piping systems (e.g. biofilms), becoming a potential hazard.

Rotavirus was reported in Kuwait by Al-Nakib *et al.* (1980). They investigated the presence of rotavirus in children in Kuwait, using electron microscopy and enzyme-linked immunosorbent assay as a non-bacterial agent causing gastroenteritis in infants.

They found it to be most commonly present in children with the disease between the ages of two to twelve months (88.1%). It was found that the virus prevalence in gastroenteritis was greater in autumn and the beginning of winter (76.1%). The most common clinical symptoms were vomiting and diarrhoea (92.9% and 76.2% respectively) (Al-Nakib *et al.,* 1980). Another detection method for rotavirus, reverse transcriptase polymerase chain reaction (RT-PCR), was utilised by Gouvea *et al.* in 1990 for the detection and typing of rotavirus in stool samples. They extracted viral RNA from either stool or tissue culture fluid, using conventional extraction methods by proteinase K and phenol-chloroform. Using conserved regions in all strains of group A rotavirus, they amplified viral RNA by RT-PCR using primers specific to gene 9 (that encodes for VP7). They found a strong correlation between the molecular and the serological method for typing rotavirus serotypes. They concluded that the detection and typing of rotavirus using RT-PCR could be used without cell culture and virus purification (Gouvea *et al.,* 1990).

1.14.5 Rotavirus replication

Although rotavirus receptors have not yet been confirmed, it is suggested that $\alpha_2\beta_1$, $\alpha_x\beta_2$, $\alpha_v\beta_3$ and $\alpha_4\beta_1$ integrins are involved in cell entry, and it was also proposed that viral entry occurs by cell membrane penetration (Figure 1.7) (Seo *et al.,* 2008). Upon viral entry, the VP4 and VP7 are lost and the virion becomes double layered, and the genetic material is released in the cell cytoplasm (Estes, 2001). Then, a transcription of the viral RNA leads to a production of many viral +sRNA, which either replicate inside the viroplasm or serve as mRNA. The viral proteins (VP1, VP2, VP3 and VP6) are the main constituents of the virion double layered particles which are also manufactured in the viroplasm, and segments of the viral genome are enclosed inside it. Finally, before cell lysis and the releases of new virions, the double layered particles gain a third layer from the rough endoplasmic reticulum of the cell (Desselberger *et al.,* 2009).

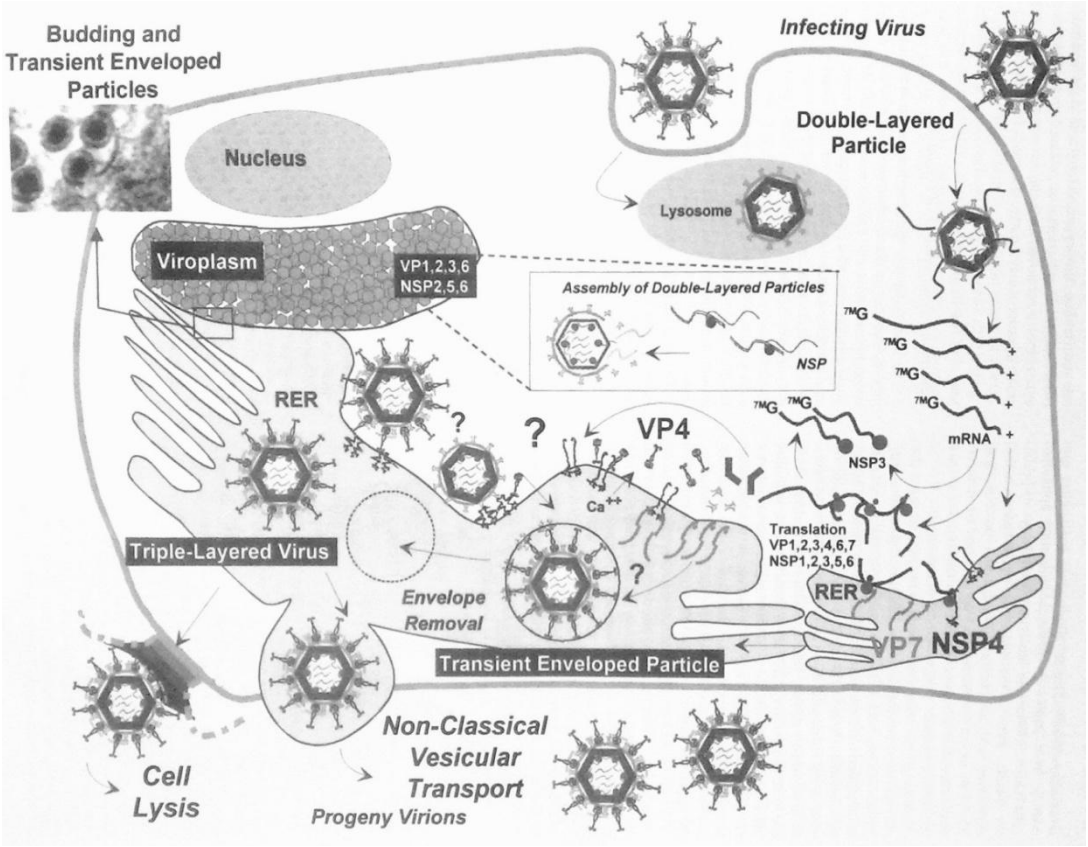


Figure 1.7. The replication cycle of rotavirus. (Quoted from Estes, 2004, p. 1767.)

1.15 Inactivation of viruses

Engelbrecht *et al.,* (1980) compared the inactivation rate of enteric viruses (polio 1 and 2, and coxsackievirus A9 and B5), using 0.4 mg/l of chlorine at different pH. The authors reported that the treatment at pH 6.0 was more effective than at pH 10.0 in viral inactivation although, again, coxsackie B5 seemed to show the greatest resistance to inactivation overall. Later on, other experimental treatments were developed to neutralise viral infectivity (Engelbrecht *et al.,* 1980). In 1985 Payment *et al.* experimented with chlorine as an agent to neutralise and inactivate poliovirus 1, 2 and 3 and coxsackievirus B4 and B5. Samples were treated with 0.4 mg/l of chlorine at 5°C and samples were taken at different time points from 1 to 100 minutes. Coxsackievirus isolates were found to be the most resistant to chlorine treatment; coxsackievirus B5 was still infective, with no change in its infectivity, even after ten minutes of treatment. Viral infectivity was 10% of the starting titre after 100 minutes of treatment, and over 0.05% was still infective after treatment for 1,000 minutes. The results suggested that coxsackievirus B5 was more resistant to chlorine treatment than B4, although

polioviruses were shown to be the most sensitive to chlorine treatment, having only 0.003% infectivity after 1,000 minutes of treatment (Payment *et al.*, 1985).

Enteric viruses are transmitted from contaminated textiles to non-contaminated ones; inactivation of enteric viruses found in either hospital or house laundry would provide higher levels of hygiene, according to Gerba and Kennedy (2007). They tested the use of sodium hypochlorite (NaOCl) to inactivate these viruses. The inactivation showed a 99.99% reduction of enteric viruses after washing, compared with the use of ordinary detergents alone (92-99%) (Gerba and Kennedy, 2007).

Inactivation of viruses by heat was shown to be effective at high temperatures; the enteric viruses were reported to be totally inactivated thermally at 90°C for three minutes (Sow *et al.*, 2010). Hepatitis A virus (HAV) was more resistant to heat inactivation which was reduced by 2.66 $\log_{10}$ difference than murine norovirus (MNV) which was reduced by 3.33 $\log_{10}$ when heated at 90°C for 90 seconds. The viruses were totally killed at the same temperature for longer duration (180 seconds). In addition, other inactivation methods require instruments and skills to achieve total inactivation, e.g. ultrasound (Su *et al.*, 2010). Total inactivation of viruses might be dependent on viral concentrations. Complete inactivation of viruses using ultrasound at 20 kHz for 30 minutes was achieved for titre of 4 $\log_{10}$ PFU/mL, while a viral titre of 6 $\log_{10}$ PFU/mL was more resistant. Viral resistance to disinfectants could be increased due to interaction with *Acanthamoeba*, as mentioned previously for bacteria.

Enteric viruses can be provided with protection from water sanitation systems that use ozone (Emerson *et al.*, 1982). Both poliovirus type 1 and coxsackievirus A19 showed recovery following their treatment with ozone when they were already internalised in mammalian cells, while they were totally inactivated at a much lower concentration of ozone (0.08 mg/l) when they were freely suspended.

Segal *et al.*, (2001) investigated the inactivation of Hepatitis C virus on Goldmann tonometer tips. The authors soaked the tips 5 minutes in either 70% isopropyl alcohol or 3% hydrogen peroxide. The authors reported that the virus was reduced by 99.98% and by 99.94% following treatment with 70% isopropyl alcohol and 3% hydrogen peroxide respectively.

In addition, Moore *et al.*, (1996) compared the inactivation of bovine immunodeffeciency virus (BIV) in milk using various temperatures. Their disinfection

experiment of milk reveled that the BIV was inactivated by pasteurisation at 72°C for 15 seconds, wheras it took 30 mintues to be inactivated at 47°C and 62°C (Moore *et al.*, 1996).

1.16 Solar disinfection (SODIS)

It has been noted that one billion people have no access to dependable sources of clean drinking water and 6,000 children die each day from dehydration resulting from diarrhoea caused by different pathogens (Meierhofer, 2006; Black, 1998). It has also been reported that different pathogenic viruses (e.g. poliovirus) can survive and remain infective for around 11 days in drinking water (Rzezutka and Cook, 2004).

It was reported that 40% of the world's population does not have water sterilization facilities. Most of these people are in developing countries and around four million people die every year, including two million children, because of water-borne diseases. In addition, only approximately 1% of all fresh water is available for use by people, with the rest in the form of ice sheets and glaciers (Parliamentary Office of Science and Technology, 2002). It is also important to mention that in the UK, for example, an estimated 96% of water used ends up as sewage or waste-water (Parliamentary Office of Science and Technology, 1998).

Earthquakes cause devastation and, besides the loss of lives, they result in the destruction of a city's water sanitation, sewage collection and treatment systems, leading to a significant shortage of safe drinking water and resulting in contaminated water. This type of incident makes people more susceptible to water-borne diseases, as evidenced following the tsunamis at Aceh, Indonesia in 2004 and 2005 respectively, which resulted in household water being contaminated with *E. coli* (Gupta *et al.*, 2007). Although other countries provide as much assistance as possible, it is often unfeasible to transfer very large volumes of safe drinking water to be used in a destroyed city. In this case, and in other similar incidents, it would be very useful to have access to technology that ensures water is safe. Solar disinfection of contaminated water, either alone or with photosensitizers like riboflavin, offers promise as it has shown faster inactivation of viruses. It is also important to mention that riboflavin is reported by FDA as Generally Regarded As Safe (GRAS), so it does not pose a hazard to the public (FDA, 2006). Due

to the problems of finding safe water and the lack of proper sanitation in developing countries such as Africa (Smith *et al.,* 2009), the low cost and ease of use of solar disinfection of contaminated water makes it a very practical and attractive prospect. In addition, this technology can help overcome the problems of water contaminated with different microorganisms, for example the water borne zoonotic parasite *Cryptosporidium*, which poses a potential health risk since it is shared between hosts, both human and animal (Fayer, 2004). Water has been reported as a significant transmitting vehicle of this pathogen.

Solar disinfection (SODIS) is a treatment using solar irradiance to inactivate or destroy pathogenic microorganisms. This method is performed by filling transparent bottles with contaminated water, followed by their exposure to sunlight for six hours. The effectiveness of the treatment is due to UV irradiance and elevated water temperature (Murinda and Kraemer, 2008). Ultraviolet light, which plays a key role in solar disinfection, is found at the end of the solar spectrum, although it is not visible to the human eye. There are three types of UV light: UV-A (near UV) with a wave length from 320 to 400nm, UV-B (Mid UV) with a range of between 290 and 320nm and UV-C (Far UV or vacuum UV) with a range between 190 and 290nm (Jagger, 1985). When UV is absorbed it leads to DNA damage by the production of reactive oxygen species (ORS) (Sichel *et al.*, 2009). Oxygen in water also affects microorganisms in the presence of light and UV, as it is reduced and gains an electron to become superoxide (O_2^-) that can damage DNA, proteins and lipids. Once it is further reduced it gains another electron to become hydrogen peroxide (H_2O_2), with an even greater oxidative effect on DNA. Moreover, when a third electron reduces H_2O_2 it results in H_2O plus a hydroxyl radical (•OH), which can result in damage and breakage of DNA strands. Gaining a fourth electron in the presence of •OH produces a second water molecule, and the reaction of •OH with $O2^-$ results in the production of singlet oxygen ($^1O_2$) (Babior, 1997; Korycka-Dahl and Richardson, 1980; Kumar *et al.*, 2004). This singlet oxygen has a major role in the inactivation of microorganisms e.g oxidation of proteins and breakage of DNA/RNA strands (Baier *et al.,* 2006; Babior, 1997; Davies, 2003).

A number of studies were performed on inactivation of bacteria using SODIS, and one of the first studies of solar disinfection SODIS was performed by Acra *et al.* (1980). In

the study an oral rehydration solution was prepared with chlorine free water and then contaminated with fresh sewage. The solution was divided into three sterile polyethylene bags, each containing one litre of oral rehydration solution. The first bag was subjected to sunlight, the second was kept in a room under artificial light, and the third was kept in the dark. The starting coliform number ranged from 7,100 to 16,500 per 100 millilitres. Under direct sunlight, the coliform number started to reduce after the first 30 minutes and all bacteria were completely inactivated after two hours. In the room with artificial light and in the dark the number of coliforms decreased by 80%, indicating the efficacy of SODIS. No re-growth of the bacteria was seen later in the sample that was under direct sunlight, suggesting that long-term storage of the solution would be possible (Acra *et al.*, 1980). Another solar disinfection study, carried out by McGuigan *et al.* (1998), showed that SODIS was able to inactivate other microorganisms, e.g. bacteria. The study used HPLC analytical reagent grade sterile water contaminated with wild type *Escherichia coli*. Using solar irradiance with a 150W xenon arc lamp, they investigated the effect of light, either in combination with temperature or alone. They found that heat was only effective at temperatures above 45°C. At 55°C, *E. coli* was totally inactivated after seven hours in combined experiment, with no recovery, indicating that heat increases the inactivation process (McGuigan *et al.*, 1998). They also reported that SODIS was able to show some inactivation of microorganisms in turbid water (200 nepholometric turbidity units = ntu). Reed and his team (2000) experimented with natural sunlight to disinfect water contaminated with bacteria in fresh samples collected from wells in India. Their experimental solar intensity was greater than 500 W/m^2, and all bacteria were killed within three to six hours. Interestingly, there was no recovery of the bacteria, even when the samples were kept in the dark for a further 24 hours (Reed *et al.*, 2000).

SODIS has also been used on encysted *Acanthamoeba* and poliovirus. A solar irradiance intensity of 850 W/m^2 was applied to samples of contaminated water at two different temperatures, 25°C and 55°C. After six hours poliovirus was completely inactivated, while *A. polyphaga* cysts were not. *A. polyphaga* is resistant and is only partially reduced in number by 3.6 logs from the starting number of 5×10^6 /ml of cysts, even at a higher temperature (50°C) (Heaselgrave *et al.*, 2006). The SODIS method has been shown to reduce the number of different pathogenic microorganisms that cause diarrhoea in children, without the need to identify each microbe (Rai *et al.*, 2010).

Using SODIS application, it can reduce the number of children suffering from diarrhoea by over 75% (Conroy *et al.*, 2001).

More recently, scientists tried to introduce chemical and mechanical enhancers to speed up and/or inactivate more pathogenic microorganisms. Fisher *et al.* (2008) attempted to improve the technique of solar disinfection of *E. coli* by introducing different additives to the contaminated water (e.g. H_2O_2, lemon, lemon juice, and copper metal or aqueous copper). This was designed to speed up the rate of inactivation of microorganisms, and to increase the effectiveness of solar disinfection in cloudy weather. They found that the introduction of these additives increased the inactivation rate of *E. coli*. However, some of the additives had limitations; for example, although H_2O_2 may be safe on its own, interactions with stabilizers in the solutions may have been problematical. Moreover, a reduction in ascorbate concentration leads to a decrease in potency as an additive, if introduced before peak sunlight is reached (Fisher *et al.,* 2008). Other enhancers like riboflavin have been found to increase inactivation of viruses in blood (Ruane *et al.,* 2004). Riboflavin (7,8-dimethyl-10-ribityl-isoalloxazine) is vitamin B2 (Figure 1.8), that dissolves in water and can be found in milk, meat, fish, certain vegetables and fruit. It contributes to redox reactions in aerobic cells, protecting against cancer and cardiovascular disease (Powers, 2003). Its efficacy and reaction mechanisms of inactivating and damaging mammalian cells, bacteriophage, bacteria, DNA and RNA have been assessed by several studies. Hoffmann and Meneghini (1979) reported that when riboflavin and tryptophan are exposed to near-UV light, hydrogen peroxide is produced, which is toxic and responsible for the breakage of dsDNA. When riboflavin is excited, oxygen reactive species (ORS) are induced, e.g. hydrogen peroxide and singlet oxygen (Besaratinia *et al.,* 2007; Cardo *et al.,* 2006; Cardo *et al.,* 2007; Hoffmann and Meneghini, 1979; Lin *et al.,* 2006; Martins *et al.,* 2008; Ruane *et al.,* 2004; Speck *et al.,* 1975 and Tsugita *et al.,* 1965). It is also known that riboflavin has a role in the metabolic processes, since it is known to be a source of essential coenzymes, e.g. flavin adenine mono- and dinucleotides (Reddy *et al.*, 2008; Steindal *et al*., 2008). However, few studies have assessed the effectiveness of riboflavin in conjunction with solar irradiation in inactivating water-borne pathogenic viruses in water.

Riboflavin is used for the treatment of keratoconus, by utilising UV-A with riboflavin to stop the progression of the keratoconus and enhance visualisation (Wollensak *et al.,* 2003). In addition, this type of treatment is simple and cheap, making it suitable for use

in poor and less developed countries. A machine called Mirasol™ PRT (CaridianBCT, USA) inactivates pathogens by employing UV-A with riboflavin to destroy their nucleic acids (Goodrich *et al.*, 2006).

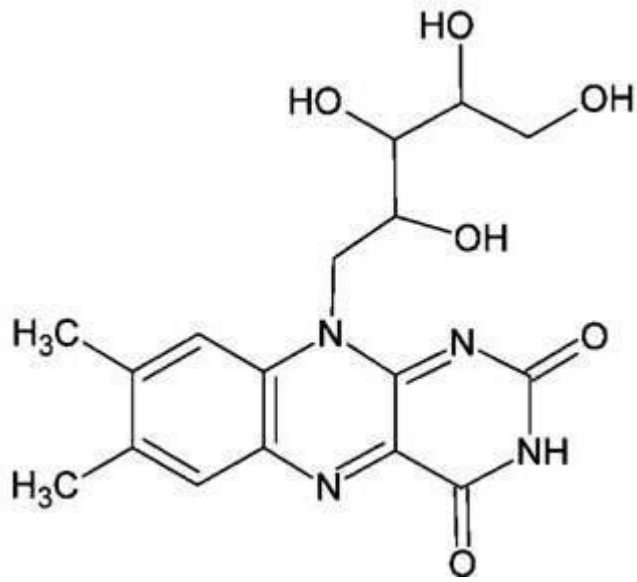


Figure 1.8. The chemical structure of riboflavin (Vitamin B2).

The use of such enhancers was applied on *Acanthamoeba* trophozoites and cysts (Heaselgrave and Kilvington, 2010). The presence of riboflavin is also effective on free-living protozoa; as expected, the *Acanthamoeba* trophozoites are more susceptible than cysts. These trophozoites are totally destroyed after four hours at 150 W/m^2, while cysts were not totally destroyed (3.5 $\log_{10}$ reduction), even after six hours of exposure to higher light intensity (250 W/m^2) and in combination with 250 µM/ml riboflavin. This inactivation method is promising and, as mentioned previously, could be suitable for providing safe drinking water worldwide, even with unskilled people. It could also be used in places where disasters have destroyed sanitation systems and safe drinking water is urgently required. The use of enhancers with SODIS might be effective in cloudy weather, which could reduce the time and the cost for the inactivation of different microorganisms present in water. Finally, this method could decrease the circulation of infectious microorganisms in the environment, thus reducing diseases worldwide.

1.17 Aims

- To investigate whether human pathogenic water-borne viruses can survive and persist in free-living protozoa. The specific questions to be addressed are: 1) Can the free-living protozoa internalise and act as a carrier for a number of water-borne pathogenic viruses (coxsackie B3N, B5F, polio 2 and rota Wa), since they share the same aquatic environment? 2) Can the free-living protozoa protect the viruses under study from harsh environments?

- To evaluate whether water treatment by solar disinfection is effective in the inactivation of viruses, both with and without free-living protozoa. Water contaminated by water-borne pathogenic viruses co-cultured with *Acanthamoeba* will be treated to find out whether the viruses are protected from solar disinfection associated with *Acanthamoeba*, making them a potential hazard in the environment. The investigation will also produce valuable data for the efficacy of solar disinfection for the inactivation of water-borne pathogenic viruses, either alone or with the assistance of enhancers e.g. riboflavin.

1.18 Objectives

- To investigate whether free-living-protozoa could be a reservoir for water-borne pathogenic viruses in the aquatic environment.
- To develop and utilise a sensitive method to detect viruses interacting with free-living protozoa.
- To establish a method for viral inactivation using a photocatalytic method (solar disinfection).
- To improve the safety of water.

Chapter Two: Materials and Methods

2.1 Materials

2.1.1 Source of Viruses

Coxsackievirus B3 Nancy strain (CVB3N), coxsackievirus B5 Faulkner (CVB5F) strain, VR-185 and Rotavirus Wa strain (RV Wa) VR-2018 were obtained from the American Type Culture Collection – Laboratory of the Government Chemist, (LGC standards), Middlesex, UK. Poliovirus type 2 strain (PV-2) was obtained from the National Collection of Pathogenic Viruses (NCPV), 503, Wiltshire, UK.

2.1.2 *Acanthamoeba*

Acanthamoeba castellanii Ma strain (ATCC 50370) was obtained from the American Type Culture Collection – Laboratory of the Government Chemist (LGC standards), Middlesex, UK, while *Acanthamoeba polyphaga* (Ros) (The strain used in this study was originally isolated from an unpublished case of *Acanthamoeba* keratitis in the UK in 1994). *Tetrahymena pyriformis* (Ehrenberg) Lwoff (1947) CCAP 1630/1W, this was obtained from the Culture Collection of Algae and Protozoa (CCAP), Scotland, UK.

2.1.3 Mammalian cell lines

The mammalian cell lines used in this study, HEp-2 cells (86030501) and African Green Monkey kidney cells MA104 (85102918) were obtained from the European Collection of Cell Cultures (ECACC), Wiltshire, UK.

2.1.4 Antibodies

Mouse anti-coxsackievirus B blend (B1-6, clone blend of 6 monoclonals) MAB9410 and mouse anti-poliovirus 2 monoclonal antibody MAB8562 were obtained from Millipore, Hertfordshire, UK. Mouse anti-rotavirus monoclonal antibody MCA2636 was obtained from Antibodies Direct Serotic, Oxford, UK and anti-mouse immunoglobulin G (IgG), whole molecule F-2012, fluorescein isothiocyanate (FITC) conjugate, which was used as a secondary antibody, was obtained from Sigma-Aldrich Ltd., Dorset, UK.

2.1.5 Chemicals

All chemicals were purchased from Sigma-Aldrich Ltd., Dorset, UK unless otherwise stated.

2.1.6 #6 basal medium

The #6 basal medium used in the study is composed of 20 g of peptone yeast extract (Biosate, UK), 5 g of glucose, 0.3 g of KH_2PO_4, 1 ml of vitamin B_{12} from stock (10 µg/ml), 5 ml of L-methionine from stock (3 mg/ml), in 1 litre of nanopure water, sterilised by autoclaving at 121°C for 15 min. If necessary, the pH was adjusted to 6.5-6.6 with 1 M NaOH.

Before using this basal medium, 1 ml of stock antibiotic (penicillin 10,000 U/ml and streptomycin 10 mg/ml) was added to 250 ml of basal medium (Hughes and Kilvington, 2001).

2.1.7 ¼ Strength Ringer's solution

The ¼ strength Ringer's solution was prepared using ready-made tablets (Oxoid, UK). One tablet was dissolved in 500 ml nanopure water in a 500 ml Pyrex bottle and autoclaved at 121 °C for 15 min.

2.1.8 Neff's encystment medium

Neff's encystment medium is composed of 0.1 M KCL, 0.008 M $MgSO_4$, 0.0004 M $CaCl_2$, 0.001 M $NaHCO_3$ and 0.02 M Tris, made up to 1000 ml and autoclaved at 121°C for 15 min (Neff *et al*, 1964).

2.1.9 Solar disinfection apparatus

Solar disinfection was performed using a Philips HPR 125W arc lamp (Philips, UK) which produced 75 W/m^2 globally of optical irradiance as shown in Figure 2.1.

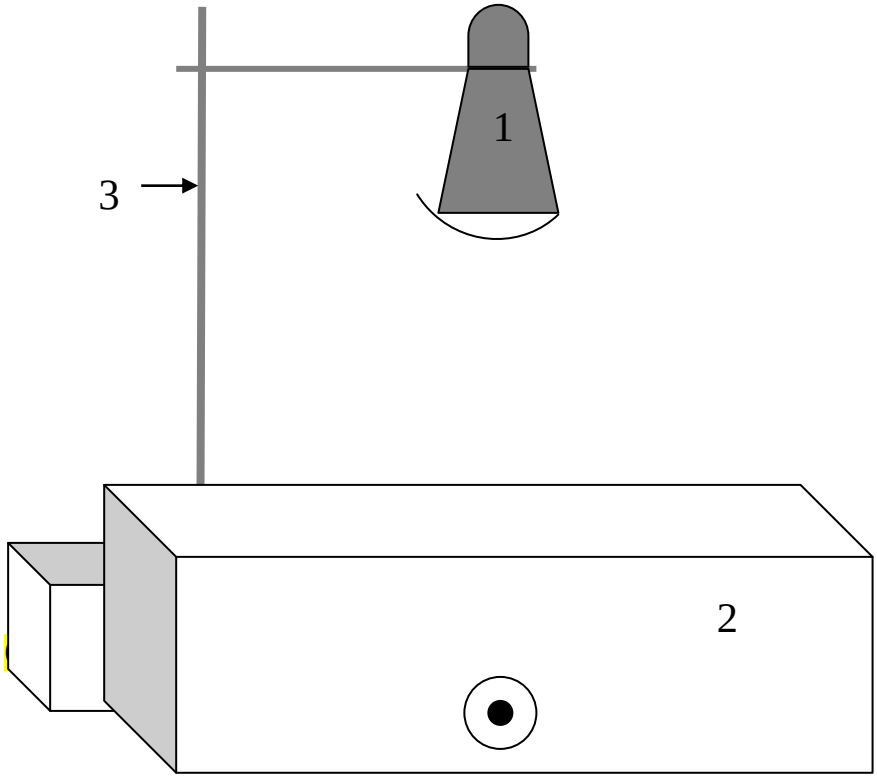


Figure 2.1. Solar disinfection apparatus: 1) 125 W/m^2 lamp having an internal reflector with integrated standard high pressure quartz mercury arc tube within its hard glass bulb, transmitting a powerful bluish-white light with strong actinic radiation; 2) water bath; 3) light stand.

2.2 Methods

2.2.1 Mammalian cell lines growth and maintenance of culture

HEp-2 cells were grown in 75 cm^2 Nunclon Cell Culture Flasks (Nunc, Fisher, UK) containing 20 ml Dulbecco's modified Eagle's Medium (DMEM, Sigma-Aldrich Ltd., Dorset, UK) or Roswell Park Memorial Institute medium 1640 (RPMI 1640, Sigma-Aldrich Ltd., Dorset, UK) with 10% heat inactivated foetal bovine serum (FBS, Invitrogen™, Paisley, UK), 2 mM of L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin final concentration. Flasks were incubated at 37°C with 5% CO_2.

MA104 cells were grown in 75 cm^2 Nunclon cell culture flask containing 20 ml Minimum Essential Medium Eagle (MEME, Sigma-Aldrich Ltd., Dorset, UK), with 10% heat inactivated FBS, 2 mM of L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin final concentraion. Flask was incubated at 37°C with 5% CO_2.

Cell lines were maintained as described generically below. Mammalian cells were grown in 75 cm^2 flasks, containing the required growth medium, which were loosely capped and incubated at 37°C with 5% CO_2 in a humid incubator (LEEC, Nottingham, UK). After 24 h, the growth medium was removed and replaced with 20 ml maintenance medium – identical to the growth medium except with a 2% final concentration of foetal bovine serum (Weller *et al.,* 1989; Schenkman and Mortara, 1992; Carthy *et al.,* 2003). The flasks were returned to the incubator and examined daily using an Olympus inverted light microscope CK2 (Olympus, Tokyo, Japan), until the cells were confluent (see Fig 2.2).

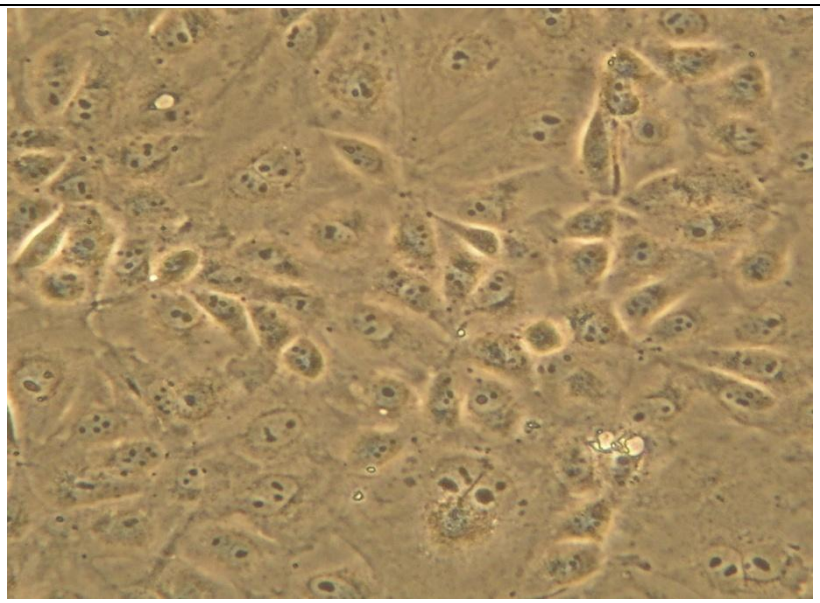


Figure 2.2. Confluent HEp-2 cells. (Magnification x400).

Cell cultures were split by detachment. The medium was first poured off and the cells were washed twice with Dulbecco's phosphate buffer saline (PBS, Oxoid Limited, Hampshire, UK), then 2 ml of porcine trypsin / EDTA acid was added. The cells were then incubated at 37°C with 5% CO_2 in a humid incubator for 2-5 min to detach the cells from the flask surface, followed by shaking and a visual check (Schenkman and Mortara, 1992). Detached cells were resuspended in 20 ml growth medium and then decanted into a 50 ml propylene centrifuge tube (Griner, Havant, UK). The suspension was centrifuged at 500 x *g* for 5 min, then the pellet was washed twice with 5 ml PBS to remove any remaining enzyme. The cells were resuspended in 1 ml fresh growth medium and counted using a Mod-Fuchs Rosenthal haemocytometer (Figure 2.3) (Hawksley, Sussex, UK). The suspension was then diluted if necessary to obtain the required cell concentration.

Figure 2.3. Mod-Fuchs Rosenthal haemocytometer, Hawksley, UK.

When the cell suspension was used for viral propagation, 20 ml of maintenance medium containing 10^5 cells /ml was used in each 75 cm^2 flask. When 25 cm^2 flasks were required, 7 ml of 10^5 cells /ml were used.

2.2.2 Acanthamoeba and Tetrahymena culture

Acanthamoeba trophozoites (*A. castellanii* and *A. polyphaga* [Figure 2.4]) and *Tetrahymena pyriformis* were grown in 75 cm^2 Nunclon cell culture flasks containing 30 ml of #6 basal medium (see section 2.1.6). The flasks were placed in a 27°C MIR 153 stationary incubator (Sanyo, Loughborough, UK) and examined daily using an inverted light Olympus microscope. When amoebal cells were needed in an experiment, amoebal cells were harvested by gently striking the side of the flask with the palm of the hand 2 or 3 times to dislodge the cells from the flask surface. The suspension was centrifuged at 500 x *g* for 3 min and then washed 3 times with ¼ strength Ringer's solution. Finally, the cells were re-suspended in 1 ml of PBS and counted using a modified Fuchs Rosenthal haemocytometer. The suspension was then diluted if necessary to obtain the required trophozoites cells concentration (e.g. 10^4/ml). Alternatively, when the cell suspension was used for propagation of the amoeba, an inoculum of the cells was used to make a new culture in 30 ml of fresh #6 basal medium in a sterile 75 cm^2 flask.

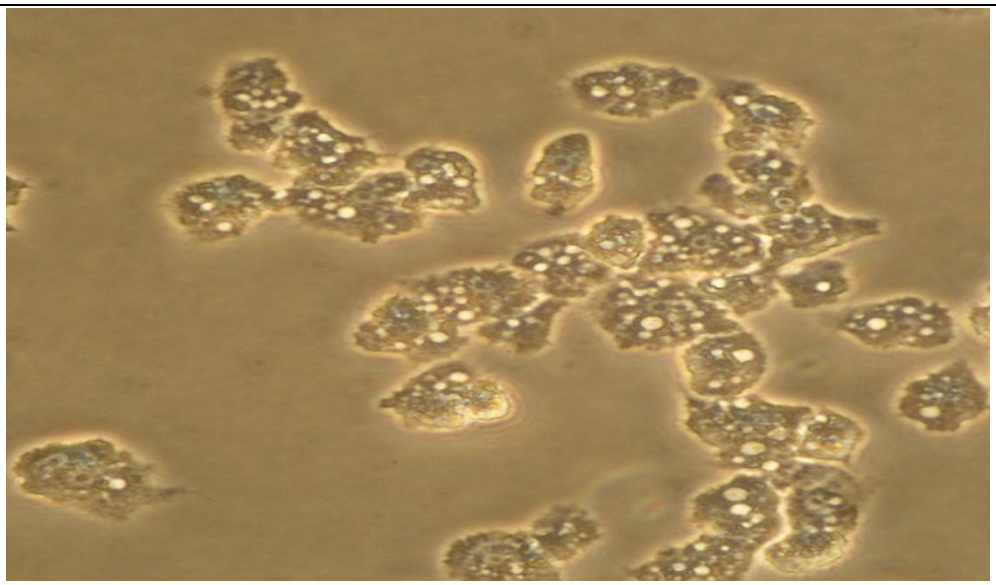


Figure 2.4. Culture of *A. castellanii* (ATCC50370) trophozoites. (magnification x400).

2.2.3 Propagation and passage of viruses

Coxsackievirus B3 (CVB3N), coxsackievirus B5 (CVB5F) and poliovirus type 2 (PV-2) were propagated on HEp-2 cells, and rotavirus WA (RV Wa) on MA104 cells. Two types of viral stocks were prepared, one having 2% FBS in the maintenance medium and the other one without FBS to see if there is any effect of FBS on viruses, except rotavirus Wa, which was always propagated in maintenance medium without FBS. This preparation is described generically as follows: once mammalian cells were confluent, the growth medium was decanted and the cells were washed twice with PBS. The mammalian cells pellet was re-suspended in 1 ml of PBS, then the cells were counted as mentioned in section 2.2.1 and a final concentration of 10^5/ml of cells was suspended in 20 ml of fresh growth medium with or without FBS. The Flask containing the mammalian cells was then incubated overnight or until the cells becames confluent. When cells were confluent in the flask, it was inoculated with 0.25-0.5 ml virus suspension (from the original bought vials of virus stocks). The culture flask was then incubated at 37°C with 5% CO_2 in a humid incubator and examined daily for the presence of any cytopathic effect (CPE, Fig 2.5, i.e. for up to 7 days or when 90% of cells are disrupted (Burleson *et al.*, 1992).

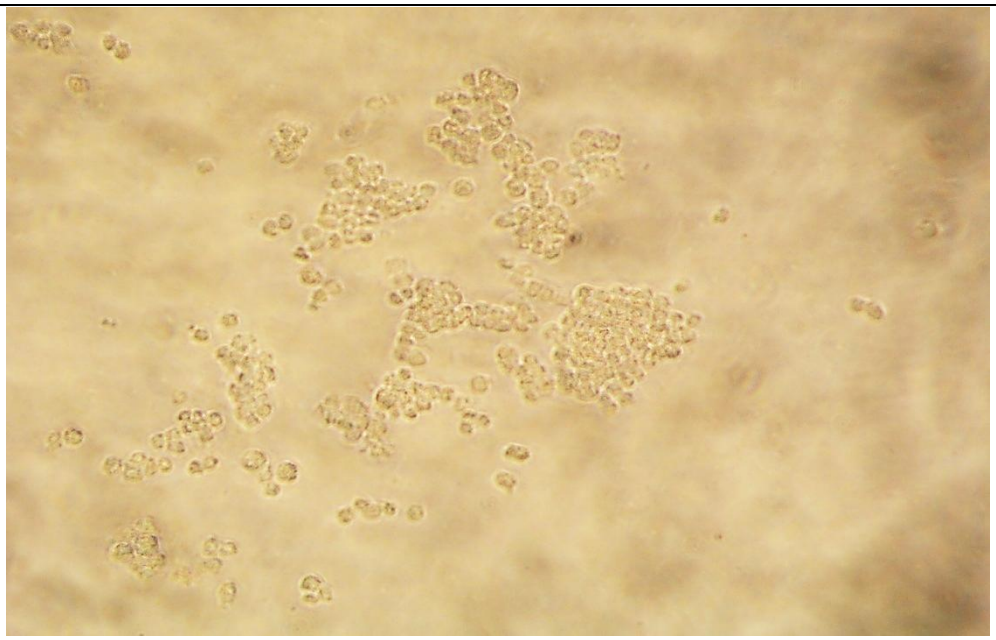


Figure 2.5. Cytopathic effect in HEp-2 cells when infected with CVB3N. (magnification x200).

When the CPE was observed (Figure 2.5), the flask was frozen and thawed twice before its contents were poured into a new sterile 50 ml centrifuge tube and centrifuged at 1000 x *g* for 10 min to remove cell debris from the virus and mammalian cells suspension.

The viral passage was performed in 75 cm^2 flask containing confluent mammalian cells, in which the growth medium was decanted and the cells were washed twice with 10 ml PBS and once with 10 ml medium alone. The virus suspension was added to the washed fresh confluent cells and incubated for 1 h at 37°C with 5% CO_2 in a humid incubator, after which the medium was replaced with 20 ml fresh maintenance medium with or without FBS, to determine the effect of FBS on viruses replication. The cells were then examined daily for the production of CPE in cells until day 7 and this process was repeated as required. When CPE was observed, the flask contents were transferred into a sterile 50 ml tube and centrifuged at 1000 x *g* for 10 min. The flask was frozen and thawed twice and the virus suspension was aliquoted into 1 ml Nalgene cryovials (Fisher, Leicestershire, UK) and stored at -80°C until further use.

2.2.4 Titration of viruses

Titration of virus suspensions was performed in 96-wells microtitre plates containing cultured mammalian cells. The plates were prepared according to the following procedure. A mammalian cells suspension (10^5/ml) was prepared as described in section 2.2.1. 90 µl of cells suspension was pipetted into each well of the 96-wells microtitre plate, which was then placed in a humid incubator at 37°C with 5% CO_2. After 24 hours of incubation, the growth medium was decanted from the wells and the cells were washed twice with 100 µl PBS. After that 180 µl of maintenance medium was added to each well.

Twenty microlitres of virus suspension was added by pipette to 4 wells of the first column in triplicate in two 96-wells microtitre plates, and the last 4 wells were the cells free of virus control. To produce a 10^{-1} dilution of the original virus suspension, a multichannel pipette was used to transfer 20 µl from the wells in the first column after mixing and added to the wells in the second column, to produce a 10^{-2} dilution of the original virus suspension. This procedure was repeated until a 10^{-12} dilution of the original virus suspension was produced. Figure 2.6 displays the final configuration of

the dilutions in the wells of the microtitre plate. The plate was then incubated at 37°C with 5 % CO_2 in a humid incubator and observed daily until day 7 for CPE using an Olympus inverted light microscope with 10x eyepiece and 20x objective.

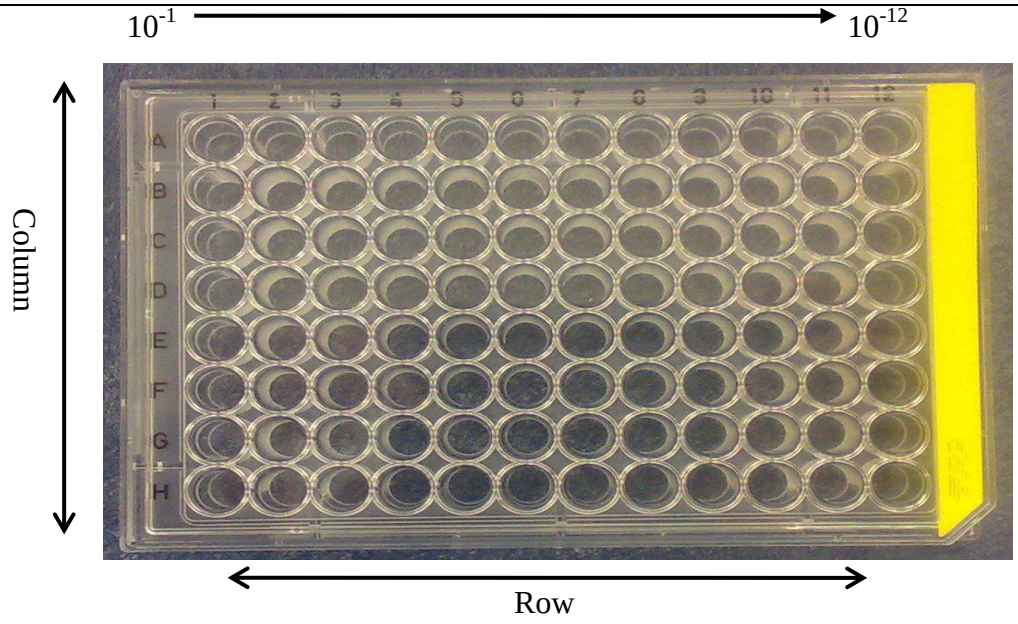


Figure 2.6. The 96-well microtitre plate used in the study

At day 7, the number of wells showing CPE in each row was recorded and then the viral titre was quantified as tissue culture infective dose ($TCID_{50/ml}$) using Spearmann-Karber method (Hamilton *et al*., 1977). The $TCID_{50}$ of a virus suspension is that dilution of the suspension which produces CPE in 50% of the cultured cell-containing wells it challenges. The $TCID_{50}$ of the suspension can then be defined as tissue culture infectious dose ($TCID_{50}$) per one ml of the suspension.

Tissue culture infective dose that are able to infect at least 50% of cells (TCID50/ml) was calculated using Spearmann-Karber method by Excel software (MS Office package), (which was kindly provided by supervisor Dr. Simon Kilvington).

2.2.5 Neutralisation of viruses

To determine which concentration of sodium hypochlorite is effective to neutralise viruses outside the amoeba cysts, an experiment of viral neutralisation was carried out for all the viruses used in this study (CVB3N, CVB5F, PV- 2 and RV Wa). Two

millilitres of each virus at a concentration of 10^7 $TCID_{50}$/ml was suspended in 5 different 15 ml tubes. To each tube, 2 ml of sodium hypochlorite was added to give final concentrations of 39, 78, 156 and 312 parts per million (ppm) of this bleach. Two millilitres of sterile nanopure water was added to the fifth tube. The tubes were incubated at room temperature for 24 h. The next day, 4 ml of 201.5 mM sodium thiosulphate was added to each tube including the controls to neutralise sodium hypochlorite, mixed gently and incubated at room temperature for 5 min. Each treated virus was then titrated against HEp-2 cells, while RV Wa on MA104 in maintenance medium in a 96-well titre plate. Plates were then incubated at 37°C with 5% CO_2 in a humid incubator and observed daily until day 7 for CPE.

2.2.6 *Acanthamoeba* treatment with sodium hypochlorite

The same procedure of viral neutralisation was used to determine the maximum concentration of sodium hypochlorite that *A. castellanii* cysts can withstand to neutralise viral particles outside the cysts. *Acanthamoeba* cysts at a concentration of 2 x 10^5/ml were suspended in 2 ml of PBS in 5 different 15 ml tubes and 2 ml of sodium hypochlorite was added to give final concentrations of 39, 78, 156 and 312 parts per million of this bleach. Two millilitres of sterile nanopure water was added to the fifth tube containing *Acanthamoeba* cysts only. The tubes were incubated at room temperature for 24 h. The next day, 4 ml of 201.5 mM sodium thiosulphate was added to each tube to neutralise the sodium hypochlorite and *A. castellanii* control, mixed gently and incubated at room temperature for 5 min. Each of the five treated *A. castellanii* cysts and control were suspended in 10 ml #6 basal medium in five different 25 cm^2 Nunc flasks and incubated at room temperature. These flasks were examined daily under light microscope to monitor the cysts for hatching.

2.2.7 *Acanthamoeba* encystment methods

Four different methods for encystment of *Acanthamoeba* with poliovirus type 2 were tested. These different techniques were performed to assess each encystment method and to decide which would be used in the experiments where virus was co-cultured with *Acanthamoeba*. These methods were: in Neff's encystment medium (Neff *et al.*, 1964),

in #6 basal medium with $MgCl_2$ (Chagla and Griffiths, 1978), in phosphate buffer saline (Mattana *et al.*, 2006) and by plating on taurine agar, as detailed below (Hughes and Kilvington, 2001).

The first *Acanthamoeba* encystment method was carried out as follows: PV-2 at a concentration of 10^6 $TCID_{50}$/ml was co-cultured with 10^4 /ml of *Acanthamoeba.* The co-culture was suspended in 10 ml of #6 basal medium in a 25 cm^2 Nunc flask and free-living protozoa free of virus were added to a second flask of the same size and the same medium to act as the control. The co-culture and control flasks were incubated at room temperature for 24 h, then the contents of both flasks were transferred to two separate 14 ml tubes and centrifuged at 500 x *g* for 5 min before being washed twice in ¼ strength Ringer's solution and a third time in Neff's medium. After the third wash, the pellet was resuspended in new Neff's encystment medium in a 25 cm^2 flask, then incubated at 30°C with shaking at 100 rpm for 5 days and examined daily until mature cysts had developed. When mature cysts were observed, they were scraped with a cotton swab to detach them from the surface of the flask, then transferred to 14 ml tubes and washed twice with ¼ strength Ringer's solution at 1000 x *g* for 5 min and resuspended in 1 ml of PBS (Hughes *et al*., 2003). Cysts were then treated with disinfectant (described in section 2.2.4 after sodium hypchlorite concentrations was assessed) by adding 1 ml of sodium hypochlorite 156 ppm (to give 78 ppm in the final concentration) for 24 h at room temperature. This step was performed to neutralise any remaining viruses in the suspension or on the surface of the *Acanthamoeba* and to destroy any remaining amoeba trophozoites or immature cysts. After this treatment, 2 ml of 201.5 mM sodium thiosulphate was added to neutralize the sodium hypochlorite, incubated for 5 min at room temperature and then centrifuged at 1000 x *g* for 5 min. The pellet was resuspended in #6 basal medium after being washed 3 times with ¼ strength Ringer's solution to remove any traces of sodium hypochlorite/thiosulphate, then incubated in a flask at 27 °C for 5 days so that the *Acanthamoeba* cysts hatched into trophozoites. After hatching, the suspension was centrifuged at 500 x *g* for 5 min and the pellets resuspended in 500 µl of sterile water. The suspensions were then frozen at -80°C for 15 min and thawed at 37°C for 5 min 3-6 times to disrupt the amoebae trophozoites, transferred to a 25 cm^2 flask containing confluent HEp-2 cells in maintenance DMEM medium and examined daily for cytopathic effects caused by virus until day 7 after transferring the broken amoebae.

The second *Acanthamoeba* encystment method involved the addition of 50 mM $MgCl_2$ to co-cultured PV-2 with *Acanthamoeba* trophozoites cells as described previously after washing 3 times in ¼ strength Ringer's solution. The mixture was suspended in 10 ml of #6 basal medium in a 25 cm^2 culture flask, as in the previous experiment, and incubated with shaking at 100 rpm at 32°C for up to 5 days or until the amoebae were seen to have matured into cysts. The suspension was then centrifuged at 1,000 x *g* for 5 min and the pellet was washed 3 times with ¼ strength Ringer's solution, as in the previous experiment. Cysts were then treated with sodium hypochlorite as in the first method for 24 h at room temperature, followed by adding sodium thiosulphate in the next day as described previously in section 2.2.6 and then washed three times with ¼ strength Ringer's solution.

Cysts were then cultured in 10 ml of #6 basal medium, incubated at 27°C and examined daily for up to 5 days until the cysts hatched. Once the *Acanthamoeba* had hatched, the suspension was centrifuged at 500 x *g* for 5 min, then the pellet was resuspended in 500 µl sterile water and the cells were disrupted as previously described by freezing and thawing. Finally, the suspensions of both the co-culture and control flasks were transferred to confluent HEp-2 cells in maintenance DMEM medium in 25 cm^2 flasks, which were incubated as in the previous method and examined daily for any cytopathic effect for up to 7 days.

The third method of *Acanthamoeba* encystment was carried out using taurine agar; two 25cm^2 flasks were used for the experiment, one contained a co-culture of PV-2 with *A. castellanii* while the second flask contained only *A. castellanii* as a control and both of them were suspended in 10 ml #6 basal medium as described previously and incubated at room temperature for 24 hours. Co-culture and control suspensions were then washed three times with ¼ strength Ringer's solution and centrifuged at 500 x *g* for 5 min. After the final wash the supernatant was decanted and replaced with new 6 ml ¼ strength Ringer's solution and vortexed for 30 seconds to distribute the trophozoites evenly in suspension. One ml of co-culture suspension was plated on each of the 6 taurine agar plates and incubated at 32°C in a stationary incubator. The virus/amoeba co-cultures and control on taurine agar plates were examined daily for cyst development under a light microscope until the cysts were developed. These were then treated with

sodium hypochlorite as in the first method for 24 h at room temperature. After treatment, cysts were washed 3 times with ¼ strength Ringer's solution and the pellet was suspended in 500 µl sterile deionised water. The same procedure for disrupting amoeba cell walls by freezing and thawing was carried out. The disrupted suspension was then added to confluent HEp-2 cells in a maintenance DMEM medium and incubated at 37°C in a 5% CO_2 humid incubator for up to 7 days.

The fourth encystment method was performed as follows: PV-2 was co-cultured with *A. castellanii* and a culture of amoeba-free virus in #6 basal medium for 24 h, as described above. The co-culture and control were then washed 3 times, twice with ¼ strength Ringer's solution and the third time with PBS solution, before being transferred to two separate 14 ml sterile tubes and centrifuged at 500 x *g* for 5 min. This was followed by suspending the pellet in 10 ml of PBS solution in 25 cm^2 flasks and incubating the suspension at room temperature for 7 days. The amoebae were examined daily for up to 7 days under a light microscope until mature cysts developed. Both the co-culture and control samples were then treated with sodium hypochlorite bleach, as previously described, to neutralise any viruses outside the amoebae in the co-culture and to kill amoeba trophozoites and immature cysts. After neutralisation of sodium hypochlorite by the addition of sodium thiosulphate to the treated cysts and wash steps, cysts were then cultured in #6 basal medium, incubated at 27°C and examined daily for up to 5 days until the cysts have hatched. The trophozoites were then disrupted by freezing and thawing as before and the disrupted cells were transferred to confluent HEp-2 cells in maintenance DMEM medium, incubated at 37°C in a 5% CO_2 humid incubator for up to 7 days and examined daily under a light microscope for any cytopathic effect.

2.2.8 Determination of the effect of amoeba on virus titre in #6 basal medium

Each of the viruses (CVB5F and PV-2) at a concentration of 10^7 $TCID_{50}$/ml was co-cultured with 10^4 /ml trophozoites of *Acanthamoeba polyphaga* (Ros) (*A. polyphaga*) in different flasks. The co-culture was set in 10 ml of #6 basal medium in a 25 cm^2 cell culture flask (Nunc, Fisher, UK) at room temperature for 5 consecutive days. One ml daily sample was taken, 500 µl of which was used for viral quantification and the remainder was used to count the amoeba cells. Viral titre measurement was carried out

by inoculating 4 wells of HEp-2 cells in maintenance medium in a 96-well titre plate with 20 µl of co-culture suspension frozen and thawed 3-6 times as described in section 2.2.7. The second part was used to quantify the number of amoeba cells in the suspension by pipette to a 1.5 ml Eppendorf tube (Eppendorf, Fisher, UK), using a Mod-Fuchs Rosenthal haemocytometer slide, described in chapter 2, section 2.2.2, to determine the effects of the interaction between the microorganisms.

The previous method of co-culture of viruses (CVB3N, CVB5F and PV-2) was used with the second amoeba (*A. castellanii*). The non-amoeba control comprised 10^7 $TCID_{50}$/ml of virus suspensions in 10 ml #6 basal medium in a 25 cm^2 Nunc flask. The non-virus control was a 10 of ml 10^4 cells/ml suspension of *A. castellanii* in a 25 cm^2 flask. All flasks were placed in a stationary incubator at 27ºC and incubated for 5 days.

2.2.9 Assessment of PV-2 internalisation in *A. polyphaga* protozoa and *T. pyriformis* by electron microscopy

Before the replication of Mattana *et al.,* (2006) method, the first experiment of virus amoeba co-culture was carried out with Free living protozoa, *Acanthamoeba polyphaga* (Ros) and *Tetrahymena pyriformis* at a concentration of 10^4/ml which were co-cultured with Poliovirus type 2 at a concentration of 10^7 $TCID_{50}$/ml in 10 ml of #6 basal medium in 25 cm^2 Nunc cell culture flask for each protozoa. Flasks were then incubated at room temperature for 48 h. Two millilitre sample of each co-culture suspension were taken at the time points 0, 24 and 48 hrs, then centrifuged at 500 x *g* for 5 min and washed 3 times with ¼ strength Ringer's solution. The pellets were then treated with 2% glutaraldehyde in PBS for 2 hrs, followed by washing the cells with 5ml of PBS before being resuspended in new 2ml PBS and taken to the electron microscopy section. Thanks to Mr. Stefan Hyman in electron microscope department for the staining by aqueous uranyl acetate of the samples which was followed by taking pictures.

2.2.10 Determination of virus internalisation by amoeba by incubating *A. castellanii* trophozoites with a freely suspended virus

This experiment was performed to replicate the protocol of Mattana *et al.,* (2006) with CVB3N, CVB5F, PV-2 and RV Wa. A suspension of *A. castellanii* was prepared as

described in section 2.2.2, but suspended in RPMI 1640 (Sigma-Aldrich, Dorset, UK). The *A. castellanii* cells were suspended in 450 µl RPMI 1640 to be 3 x 10^6 cells final concentration in 500 µl in a 10 ml polystyrene round bottom tube (Becton Dickinson, Oxford, UK). Virus / amoeba co-cultures were made at a ratio of 1:3, by adding 50 µl of virus suspension to be 10^6 $TCID_{50}$ final concentration in the 500 µl total volume. The non-virus control was 3 x 10^6 final concentration of *A. castellanii* cells in 500 µl RPMI 1640 (450 RPMI 1640 plus 50 µl of distilled sterile water) in a 10 ml polystyrene round bottom tube. Tubes of co-culture and control were placed into a stationary incubator at 27ºC. Centrifugation of co-culture suspension at 100 x *g* for 5 min was performed 1 h post co-culture, this was followed with 3x washing with 8 ml PBS. The pellet was resuspended in 1 ml of RPMI1640, 50 µl of FBS and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin final concentrations) and incubated at 27°C. After 1 and 24 hrs of incubation an aliquot of 250 µl sample of each tube was transferred by pipette to a new sterile 1.5 ml Eppendorf tube and centrifuged at 100 x *g* for 5 min. The pellet was then resuspended in 250 µl PBS buffer which was used for indirect immunofluorescence examination (section 2.2.13). A second 250 µl of the co-culture was frozen and thawed 3-6 times and poured on mammalian cells in maintenance medium in 25cm^2 flask which was incubated at 37^{o}C in 5% CO_2 humid incubator. The flask was monitored daily for the development of CPE in mammalian cells. The rest of the 1 ml co-culture suspension was stored at -80^{o}C. In due course, the sample was removed from the freezer and held at room temperature until completely thawed. An aliquot of 140 µl was then used for RNA extraction and subsequent RT-PCR as described in sections 2.2.14 and 2.2.15 to detect viral RNA. All tests were performed in triplicate.

2.2.11 Co-culture of *A. castellanii* trophozoites with virus-infected mammalian cells

This experiment was performed with each of the viruses (CVB3N, CVB5F, PV-2, and RV Wa). Mammalian cells were grown to confluence in 25 cm^2 flasks, the growth medium was discarded and the cells were washed twice with 5 ml PBS. Seven ml of serum-free RPMI 1640, 2 mM L-glutamine and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin final concentrations) were added to the confluent mammalian cells, followed by adding 100 µl of 10^4 $TCID_{50}$/ml virus suspension to produce CPE slowly.

The flasks of mammalian cells inoculated with virus suspension were then incubated at 37^{o}C of 5% CO_2 humid incubator for 18 hrs or by the time >25 % of cells had developed a CPE. Following the CPE development, *A. castellanii* 2 x 10^4 /ml suspension (to feed slowely on mammalian cells) was washed twice with 5 ml of ¼ strength Ringer's solution, then the pellet was resuspended in 1 ml RPMI 1640 and added to the flask of mammalian cells infected with viruses and then flasks were returned back to the incubator.

After 18 hrs of incubation or the development of >25 % CPE in mammalian cells, one ml of the contents was removed by pipette to a 1.5 ml microtube, the suspension was washed twice with PBS and used for indirect immunofluorescence as described in section 2.2.13. To determine if infectious viruses were internalised in *A. castellanii,* 5 ml of the virus-infected mammalian cells co-cultured with *A. castellanii* was transferred by pipette to a new sterile 15 ml tube and frozen/thawed 3-6 times. The disrupted cells were then inoculated on confluent mammalian cells in maintenance medium in 25cm^2 flask at 37°C CO_2 humid incubator. The flasks were monitored daily for the development of any cytopathic effect as described in section (section 2.2.6), the rest of the infected mammalian cells/*Acanthamoeba* co-culture was stored at -80^{o}C. In due course, the sample was removed from the freezer and held at room temperature until completely thawed. An aliquot of 140 µl of the co-culture frozen suspension was then taken for RNA extraction and subsequent RT-PCR to detect the presence of the viral RNA as described in sections 2.2.14 and 2.2.15 to detect viral RNA. The experiment was performed in triplicate.

2.2.12 *Acanthamoeba* encystment following co-culture with viruses

This experiment was performed for the co-cultured CVB3, CVB5, PV-2 and RV Wa with *A. castellanii* in PBS or Neff's encystment medium (section 2.2.7). Virus/*A. castellanii* suspensions and their respective controls were prepared as described in section 2.2.10. All flasks were placed in a stationary incubator at 27ºC and incubated for 24 hrs. After 24 hrs the flaks contents (30 ml) were centrifuged at 500 x *g* for 3 min and the supernatant was discarded. The pellet was then washed twice with 8 ml PBS if encysting in PBS and the third wash with encystment medium of Neff (when encystment of *A. castellanii* in Neff's encystment medium) (section 2.2.5). Finally, the

pellet was resuspended in 30 ml Neff's encystment medium or 30 ml PBS and put into a 75 cm^2 flask, which was then incubated for up to 7 day or until mature cysts were developed in an orbital incubator (Sanyo, UK) at 30^oC with shaking at 100 rpm or at room temperature when looking for encystment in PBS. The trophozoites were examined daily by inverted light microscopy until mature cysts were observed. When the majority of trophozoites ≥ 90% had encysted, the cysts suspension was centrifuged at 1000 x *g* for 10 min and the supernatant was discarded. Following centrifugation, the pellet was then washed twice with PBS and finally suspended in 1 ml PBS. One millilitre of 156 ppm sodium hypochlorite was added (Milton bleach, Milton Pharmaceutical Ltd, France) to give a final concentration of 78 ppm and incubated for 24 hrs at room temperature, this treatment was used to kill immature cysts and to inactivate viruses outside amoeba. Subsequently, 2 ml of 201.5 mM sodium thiosulphate was added to neutralise the sodium hypochlorite and the cyst suspension was then incubated at room temperature for 5 min. The suspension was centrifuged at 1000 x *g* for 10 min and the supernatant was discarded. This was followed by washing 3 times with 10 ml PBS and finally suspended in 10 ml #6 basal medium. The cysts were then incubated at 27°C for 7 days for *Acanthamoeba* to hatch. The hatching of the majority > 90% of cysts was confirmed by examining for trophozoites using inverted light microscopy. When hatching was confirmed, 5 ml aliquot was centrifuged at 500 x *g* for 5 min, the supernatant was discarded, followed by suspending the pellet in 500 µl sterile water and frozen / thawed 3 to 6 times. The suspension was then inoculated on confluent mammalian cells in a 25 cm^2 flask containing 7 ml of serum-free growth medium. The flask was incubated at 37°C in a 5% CO_2 humid incubator and examined daily up to 7 days for the presence of CPE. The rest of the 10 ml #6 basal medium co-culture was stored at -80^oC. In due course, the remainder of the 10 ml cell suspension was removed from the freezer and held at room temperature until completely thawed. An aliquot of 140 µl was then taken for RNA extraction and subsequent RT-PCR as described in sections 2.2.14 and 2.2.15. The experiment was performed in triplicate.

2.2.13 Detection of viruses by indirect immunofluorescence

Before viral particles were used in the co-culture with *A. castellanii*, mammalian cells were infected and the presence of virus was confirmed using indirect

immunofluorescence with specific anti-virus antibodies and their binding was detected using fluorescent labelled anti-mouse antibodies. The optimum dilution of antibodies was determined by using serial dilutions of viral antibody in the range of 1:50-1:51200 (antibody:PBS) for enterovirus antibodies and 1:10-1:100 (antibody:PBS) for RV Wa antibody using mammalian cells infected with the viruses. An inoculum of 20 µl of 2 x 10^4 $TCID_{50}$ /ml of CVB3N, CVBF5, PV-2 was added to confluent HEp-2 cells in maintenance medium in 12 wells of a 96 well microtitre cell culture plate and the second 12 wells were not inoculated to act as controls (non-infected cells). While for RV Wa, an inoculum of 3 ml RV Wa (from RV Wa passage flask) was inoculated on MA104 cells in 4 ml MEME in 25 cm^2 flask for 24 hrs or until ≥ 25 % of mammalian cell showed CPE. The flask contents were centrifuged, followed by resuspending in 2 ml PBS and seeded on 6 wells of 2 chamber slides (12 wells). The fixation and indirect immunofluorescence procedures for RV Wa were performed as for the other viruses with specific antibodies. Once a ≥ 25% cytopathic effect was observed in the cultured Hep-2 cells the medium was aspirated using a manual multichannel pipette and 100 µl of PBS was used to wash the cells and remove the medium, this step was repeated twice. After the PBS was aspirated, infected cells and controls (non-infected mammalian cells) were then fixed for 45 min with 200 µl freezing methanol at -20^oC. The wells were left to dry for ≤ 5 min after the aspiration of methanol and 100 µl of primary mouse anti-virus antibody (1:50 antibody:PBS) was pipetted into the first well, while 50 µl of water was pipetted into wells 2-12. An aliquot of 50 µl of the primary antibody stock was diluted by taking 50 µl of the stock which was then serially diluted in the next 11 wells of the fixed mammalian cells infected with viruses (CVB3N, CVB5F and PV-2) and the non-infected cells. While for RV Wa, primary antibody diluted in PBS (neat, 1:10, 1:20, 1:30, 1:40 and 1:50) was prepared in 0.2 ml PCR tubes and was added to each defined well of infected and non- infected cells. Both the 96-microtitre plate and the chamber slide were incubated at room temperature in the dark for 1 hr. Next, the primary antibody was aspirated and infected cells and controls were washed 3 times by adding 100 µl of PBS containing 0.5 % Tween™ 80 %, which was left for 5 min. to remove any unbound antibody and aspirated as previously mentioned in this section. After the third wash, an aliquot of 50 µl of secondary fluorescein isothiocyanate (FITC) labelled anti-mouse antibody diluted in PBS (1:64 antibody:buffer) was added to each well of infected mammalian cells and controls (non-infected cells) which were incubated with or without primary antibody at room

temperature for 1 hr in the dark. Following incubation with the secondary antibody the cells were washed in the same way as for the primary antibody to minimise background staining. Finally, after the third wash had been aspirated, the wells were loaded with one drop of anti-fade mounting buffer (0.233 g of 1,4-diazabicyclo[2.2.2]octane (DABCO), 200 µl of Tris-HCl 1 M pH 8.0, 800 µl sterile water and 9 ml glycerine (86%)) and examined directly under an immunofluorescence microscope using FITC filter. If stained viruses were not examined on the same day, plates were stored at 4°C in a refrigerator and examined within one week of immunofluorescence staining.

Indirect immunofluorescence staining for the presence of viruses (CVB3N, CVB5F, PV-2 and RV Wa) within *A. castellanii* co-cultured was carried out in the same way as for mammalian cells already infected with each of the viruses separately (positive controls), except that a second immunostaining with primary and secondary antibodies was performed in chamber slides for all the staining steps. Suspensions of co-cultured *A. castellanii* and control samples were then centrifuged at 300 x *g* for 5 min and the pellet was resuspended in sterile PBS buffer, transferred into chamber slide wells and incubated at 27^{o}C for 1 hr. Fixation of cells was performed as described above for positive controls. Primary and secondary antibody was then added and incubated as above. Immunofluorescence detection was performed as described above.

2.2.14 Extraction of viral RNA

Total viral RNA of CVB3N, CVB5F, PV-2 and RV Wa was extracted using the QIAamp® Viral RNA Mini Kit (QIAamp® Viral RNA Mini Kit 52904, Qiagen, West Sussex, UK) following the protocol of the manufacturer as follow: 140 µl of viral suspension was added to 560 µl of buffer AVL-carrier RNA (contains guanidine thiocyanate) and pipetted into a 1.5 ml sterile Eppendorf tube, then vortexed for a few seconds. The mixture was incubated at room temperature for 10 min. Next, 560 µl of 100 % ethanol was added to the mixture and vortexed for few seconds, then 630 µl of the mixture was transferred to the QIAamp mini column, which was fitted into a 2 ml collection tube and centrifuged at 6000 x *g* for 1 min, this step was repeated twice. Next, 500 µl of buffer AW1 was added and the tube centrifuged at 6000 x *g* for 1 min.

The QIAamp mini column was then placed in a clean 2 ml collection tube to which 500 µl of AW2 buffer was added and centrifuged at 13,000 x *g* for 3 min. Finally, to elute the RNA, 60 µl of AVE buffer was pipetted into the QIAamp mini column after it was placed in a new sterile 1.5 ml microcentrifuge tube. After incubation at room temperature for 1 min it was centrifuged at 6000 x *g* for another minute. At the end of this extraction process, the RNA was stored at -20°C until further use.

2.2.15 Detection of viral RNA by reverse transcription polymerase chain reaction (RT-PCR)

Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed to detect each viral RNA type with specific primers added to the RT-PCR mixture with the exception of poliovirus type 2, for which universal primers were used. The primers were used to detect CVB3N (GenBank accession number M16572.1) targeted sequences that include VPg and protease gene, which was expected to produce an amplicon size of 521 bp. Both sense and antisense primers were used to detect CVB5F (GenBank accession number AF114383.1) targeted sequences includes part of the polymerase genome was expected to produce an amplicon size of 760 bp. Both CVB3N and CVB5F primers were designed online at the GeneFisher website (http://bibiseRV.techfak.unibielefeld.de/genefisher2/submission.html). In addition, primers used to detect PV-2 (vaccine derived poliovirus) targeted the start before and after VP4 (GenBank accession number DQ890385.1, previously purchased by the laboratory) and the expected amplicon size was 650 bp and the position of the amplified region extends across the VP4 region. Finally, before reverse transcribing, RV Wa (GenBank accession number K02033.1) RNA was incubated at 95°C for 5 min to separate the double-strand RNA. The primers used for detection of RV Wa genome targeted segment 9 that encodes the outer capsid glycoprotein VP7 (Gouvea *et al.*, 1990) and the amplicon size expected to be produced was 1062 bp. All primers were obtained from Operon, Germany. All primer sequences are shown in Table 3.1.

Table 2.1. Oligonucleotide primers used in this study

Virus	sequence (5′ - 3′)	Orientation	Tm	Reference
Coxsackievirus B3 Nancy VP4	GCTTATACAGGAGTGCCCAAC	Forward	62.57 °C	This study
	GACATGAGCACTCCACCACAC	Reverse	64.52 °C	
Coxsackievirus B5 Faulkner VP4	GAACCAGCTGTCCTCAGA	Forward	59.9 °C	This study
	TAGCCCACCCATCATAGAACAT	Reverse	60.81 °C	
Poliovirus type 2 VP4	GCATCIGGYARYTTCCACCACCANCC	Forward	69.32 °C	(Olive *et al.*, 1990)
	GGGACCAACTACTTTGGGTGTCCGTGT	Reverse	69.16 °C	
Rotavirus Wa VP7	GGACCAAGAGAAAACGTAGC	Forward	60.4 °C	(Gouvea *et al.*, 1990)
	GGTCACATCATACAATTCTAATCTAAG	Reverse	60.05 °C	

I= inosine; Y=T, C; R=G, A; N=A, G, C, T)

RT-PCR detection was performed using the Reverse-iT One Step Kit (0844, AB-gene, Epsom, UK). RT-PCR mixture was prepared with 12.5 µl of 2 x RT-PCR Master Mix (Thermoprime Plus DNA polymerase 2.5 U/25 µl, $MgCl_2$ 1.5 mM) Optimise reaction buffer, dNTPs at a final concentration of 0.4 µM, of each of sense and antisense primers at a final concentration of 0.5 µM, 0.5 µl of Reverse-iT™ enzyme RTase Blend (50 U/ µl) (including RNase inhibitor), 2 µl of RNA template and completed to 25 µl with RNase/DNase-free water in a 0.2 ml Eppendorf PCR tube. The RT-PCR protocol was applied for all the viruses in the study which was performed as follows:

Reverse transcription	42ºC	for	45 min	
Denaturation	95ºC	for	3 min	
Denaturation	95ºC	for	30 sec	35 cycles of
Annealing	58ºC	for	45 sec	
Extension	72ºC	for	1 min	
2 holds				
Final extension	72ºC	for	5 min	
	14ºC	for	∞	

2.2.16 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to separate the RT-PCR DNA products on the basis of their molecular size. Since DNA has a negative charge at neutral pH, the DNA product moves to the positively charged anode when an electrical field is applied. Gel electrophoresis was performed in TBE buffer (Tris-base, boric acid and 0.5 M EDTA, pH 8). A 1.2% agarose gel (Bio-rad, UK) was dissolved in TBE buffer and then molten using the microwave. The gel was then poured in a plastic tray containing a comb to provide the required number and size of wells and left at room temperature until the gel solidified. The gel was then transferred to an electrophoresis tank. The RT-PCR amplified DNA was mixed with x2 bromophenol blue loading buffer before loading the samples in the gel wells. The electrophoresis was carried out at 90V for 45 min, then the

gel was viewed on a UV transilluminator (Geneflow, UK) and photographed using an Olympus Zoom camera (C-5050, Olympus, Tokyo, Japan).

2.2.17 Sensitivity test of RNA detection

Prior to the detection of viral RNA for all viruses, RNA concentration sensitivity test was performed to determine the minimum RNA concentration that can be visulised by gel electrophoresis following its amplification using RT-PCR. The test was applied using as previously mentioned (section 2.2.15) volumes and concentrations of all RT-PCR regents of the tested viruses but with viral concentrations (10^7, 10^5, 10^3, 10^2 and 10 $TCID_{50}$/ml).

2.2.18 Sequencing of DNA

The RT-PCR product DNA of each virus was purified by Montage® PCR Cleanup Kit (Millipore, Hertfordshire, UK) followed by sequencing by PNACL at the University of Leicester. The amplified and sequenced DNA was read by chromas program (v. 2.01 Technelysium Pty Ltd) and analysed using The Basic Local Alignment Search Tool (BLAST) at the http://blast.ncbi.nlm.nih.gov/blast.cgi website, which confirmed that the detected viral strains were those used in the experiments.

2.2.19 Solar disinfection of contaminated water with viruses

This experiment was performed with CVB3, CVB5 and PV-2 viruses. A suspension of each virus was prepared according to section (for virus concentration only) 2.2.8 of chapter 2, and then adjusted in 12 ml sterile deionised water to a concentration of 5×10^8 $TCID_{50}$/ml. Two millilitres were pipetted into each of the 3 wells of the two 12-well polystyrene capped plates (Triple Red Laboratory Technology, Oxfordshire, UK). The control plate was covered with aluminium foil and the other left uncovered. Both plates were placed floating on water at 30°C in the JB1 water bath (Grant Instrument Ltd, Cambridge, UK) and subjected to solar irradiance 75 W/m^2 for 6 h. At times 0, 1, 2, 4 and 6 h, each plate was removed from the water bath and gently agitated by hand to mix the viral suspension, then 20 µl was removed from each well and used immediately for virus titration following the procedure described in section 2.2.4. All tests were performed in triplicate.

2.2.20 Solar disinfection of water contaminated with viruses in the presence of riboflavin

This experiment was performed with CVB3, CVB5 and PV-2. A virus suspension was prepared according to section 2.3.1 and titred according to section 2.2.4, then diluted in 12 ml sterile deionised water to a concentration of 5×10^8 $TCID_{50}$/ml. This was done in duplicate in 50 ml tubes. To the first tube, riboflavin was added to make a final concentration 250 µM (50 mM stock), while the second tube was without riboflavin. The experiment was conducted in 3 wells of 2 rows in 12-well polystyrene cell culture plates. Two ml of the virus suspension was pipetted into each of a 3 wells in a row and 2 ml of the virus suspension plus riboflavin was pipetted into a second row of 3 wells. One plate was covered with aluminium foil as a control and the other was left uncovered, then the experiment proceeded as described in section 2.2.19.

2.2.21 Solar disinfection of water contaminated with viruses in the presence of *A. castellanii*

This experiment was performed with CVB3, CVB5 and PV-2. One millilitre of *A. castellanii* 10^4 /ml was co-cultured with 1 ml of $5x10^8$ $TCID_{50}$ /ml of each of the viruses and the controls (virus alone), as in section 2.2.10 but in RPMI 1640 (similar to Mattana *et al.,* 2006 medium). All of the tubes containing viral / amoeba suspensions were placed into a stationary incubator at 27°C and incubated for 24 h. The suspension was then pipetted into 50 ml tube and 12 ml sterile deionised water was added to it. From each tube 2 ml was pipetted into one 3 well row of two 12-well polystyrene capped plates. One plate was covered with aluminium foil and the other was left uncovered, then the experiment proceeded as described in section 2.2.20.

2.2.22 Solar disinfection of water contaminated with viruses in the presence of *A. castellanii* and riboflavin

This experiment was performed with CVB3, CVB5 and PV-2 as follows; one millilitre of virus / *A. castellanii* suspension was co-cultured with 1 ml of $5x10^8$ $TCID_{50}$ /ml of each of the viruses and was prepared as in section 2.2.10 and the respective controls were prepared in RPMI 1640 as described in section 2.2.10. Each suspension was placed in a stationary incubator at 27 °C and incubated for 24 h. This was done in duplicate in 50 ml tubes and 12 ml sterile deionised water was added to it. To this suspension of co-culture riboflavin was added to it (stock 50mM) to make the final concentration 250 µM in one tube, while the second tube was without riboflavin and the experiment proceeded as described in section 2.2.20.

2.3 Data analysis

The p values for all the experiments were computed using student t test in GraphPad Prism software version 5.01, August 2007.

The total optical dose (KJ) was calculated using the equation "Power (kilowatts) x time (seconds) = kilojoules" (Quoted from: Bird, 2007. In: Electrical and Electronic Principles and Technology. pp.4).

Chapter Three: Results

3.1 Co-culture and Immunofluorescence

3.1.1 Electron microscopy of poliovirus co-cultured with free-living protozoa

Electron microscopy of *A. polyphaga* (Ros) or *T. pyriformis* co-cultured with poliovirus (PV-2) did not show viral particles present in amoebae samples taken at T0h, T24h and T48h of the co-culture (Figures 3.0 A-C) when compared with the positive control PV EM pictures (Figures 3.0 D-G). After collecting and washing the amoeba from these co-cultures, the amoebae were freeze-thawed 3-6 times and extracts were added to confluent mammalian cells in maintenance medium and incubated at 37°C in a humid CO_2 incubator for 7 days. No cell CPE was observed, supporting the evidence that no infective virus had been internalized or attached to the surface of the free-living protozoa in these experiments (data not shown).

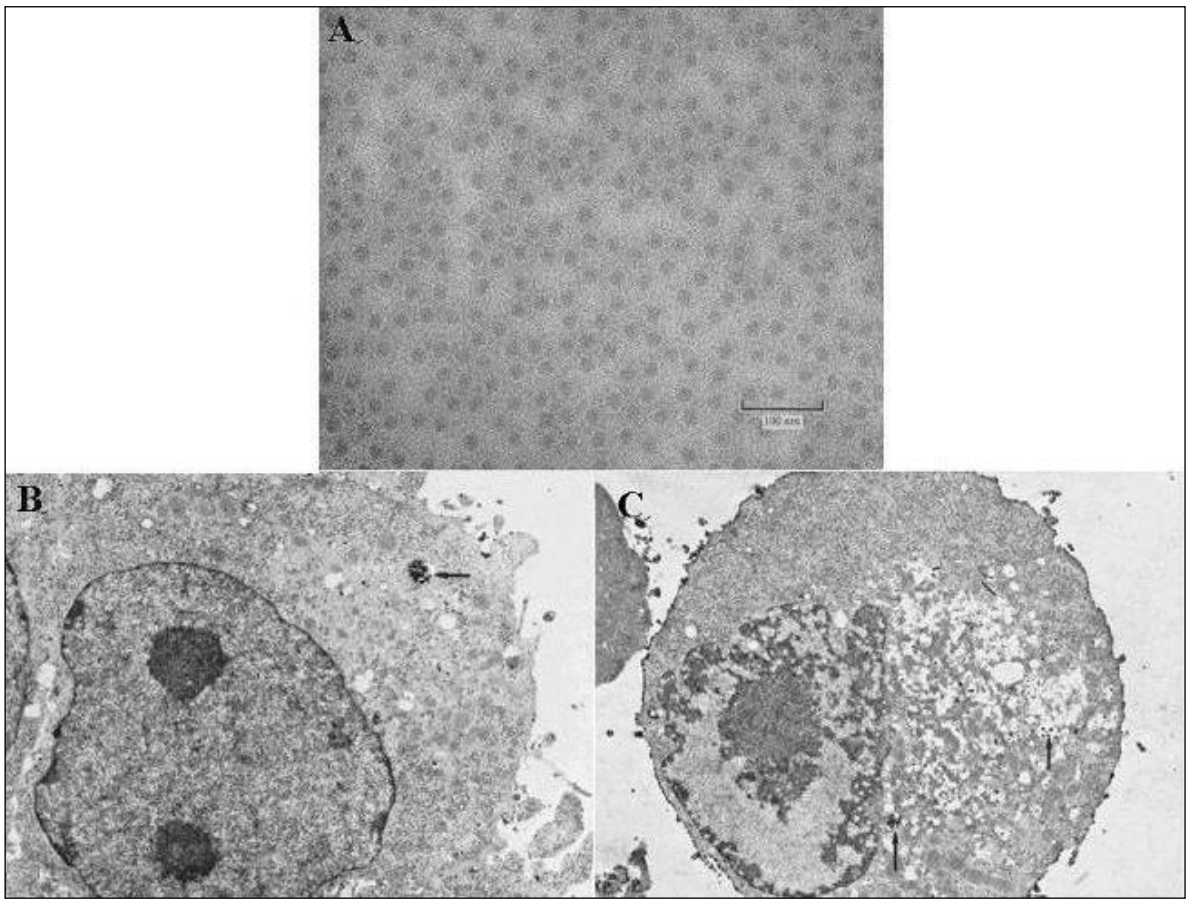


Figure 3.0M. Electron microscopy of poliovirus A) poliovirus (x100nm), B) HEp-2 cell 2h post-infection with poliovirus (x10,500), C) Hep-2 poliovirus 5h post-infection with poliovirus (x9,200) (Picture A is quoted from Boublik and Drzeniek, 1976, Picturess B and C quoted from Heding and Wolff, 1973).

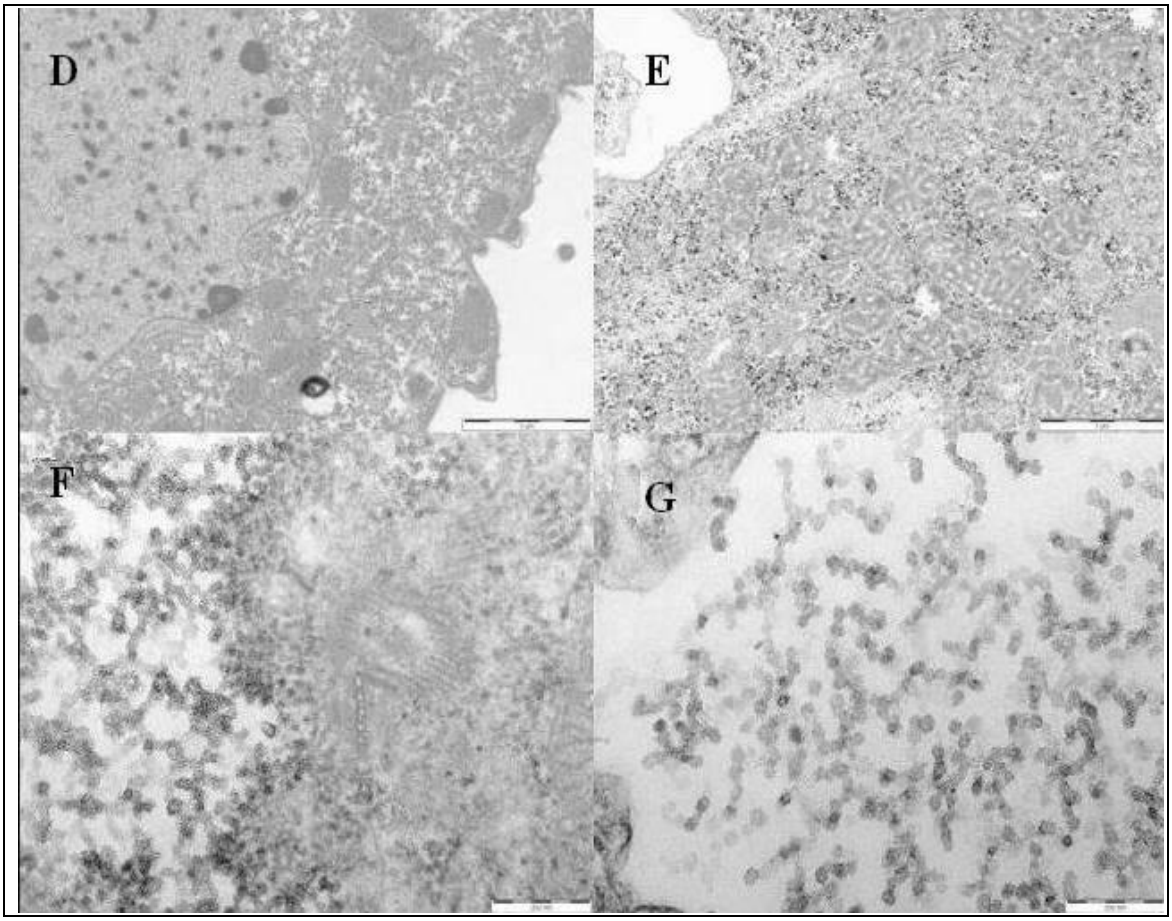


Figure 3.0N. Electron microscopy of A) *T. pyriformis* 0h post co-culture with PV-2 (magnification x8500), B) *T. pyriformis* 24h post-co-culture with PV-2 (magnification x70000), C) *A. polyphaga* 0h post-co-culture with PV-2 (magnification x16000) and D) *A. polyphaga* 24h post-co-culture with PV-2 (magnification x60000).

3.1.2 Viral titre measurement

CVB3N, CVB5F and PV-2 were propagated in HEp-2 cells, while the RV Wa viral stock was propagated using the MA104 cell line. In an initial experiment, it was found that the titre of the CVB3N virus was 10^8 $TCID_{50}$/ml, CVB5F was 3 x 10^8 $TCID_{50}$/ml and PV-2 was 5.6 x 10^7 $TCID_{50}$/ml, propagated in mammalian cells containing 2 % FBS in DMEM. The viruses propagated in mammalian cells in maintenance medium without FBS showed a higher titre than with FBS for CVB3N, CVB5F and PV-2: 1.8 x 10^9, 2 x 10^9 and 1.4 x 10^9 $TCID_{50}$/ml respectively (Table 3). However, RV Wa did not show a good viral CPE on MA104 cells, even when repeated more than three times on different occasions and following different published protocols (such as ATCC protocol, 2005; Almeida and Hall, 1978), yielding less than 10 $TCID_{50}$/ml, although it arrived as 10^8 $TCID_{50}$/ml from ATCC. Although the titration experiments with RV Wa were

unsuccessful, the virus did show good CPE on passage (> 6 times) in tissue culture, with 80% lysis of MA104 cells within 24-72 h. In addition, it was successfully detected by indirect immunofluorescence using a specific RV Wa mouse anti-rotavirus Wa antibody (Figure 3.23) and its RNA was detected by RT-PCR using strain specific primers (Gouvea *et al.*, 1990) which showed clear and bright bands (Figure 3.36).

Table 3.1. Titres of viruses ($TCID_{50}$/ml) with and without Foetal Bovine Serum (FBS) (n = 3).

	Titre with FBS			**Titre without FBS**		
Virus	CVB3N	CVB5F	PV-2	CVB3N	CVB5F	PV-2
Titre	10^8	$1.8\ 10^8$	5.6×10^7	1.8×10^9	10^9	5.6×10^8
	$1.8\ 10^8$	$1.8\ 10^8$	5.6×10^7	5.6×10^8	1.8×10^9	1.8×10^9
	$3.2\ 10^7$	5.6×10^8	5.6×10^7	3.1×10^9	3.1×10^9	1.8×10^9
Mean	10^8	3×10^8	5.6×10^7	1.8×10^9	2×10^9	1.4×10^9

3.1.3 The effect of viral/amoeba co-culture on viral titre and cell toxicity

As described in section 2.2.8, the experiment, which ran for 5 consecutive days in #6 basal medium, figure 3.1 shows the titre of poliovirus type 2 following co-culture with *Acanthamoeba polyphaga* (Ros) at room temperature, showing that *Acanthamoeba* had no effect on poliovirus number (t test $P \geq 0.3$). Along with the titre measurement, the number of *Acanthamoeba* was quantified to study the effect of viruses on the survival of cells in the co-culture (Figure 3.2). There was no effect of poliovirus on *Acanthamoeba* cell survival (t test $P \geq 0.8$) indicating that *Acanthamoeba* was growing normally, compared to amoeba control virus-free. Log Nt/N0 where N0 = the original cell count or virus titre while Nt = the number of cells or virus titre at time t)

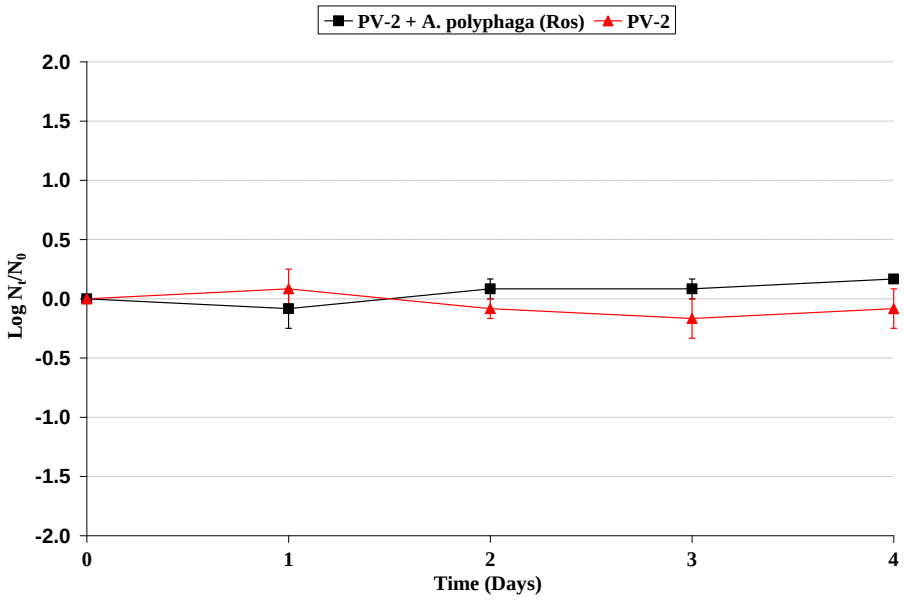


Figure 3.1. Titre of poliovirus type 2 (PV-2) either alone ( ) or co-cultured with *A. polyphaga* ( ). There was no significant difference in the titre of PV-2 alone or when cultured with *A. polyphaga* (Ros) ($P \geq 0.3$), n=3.

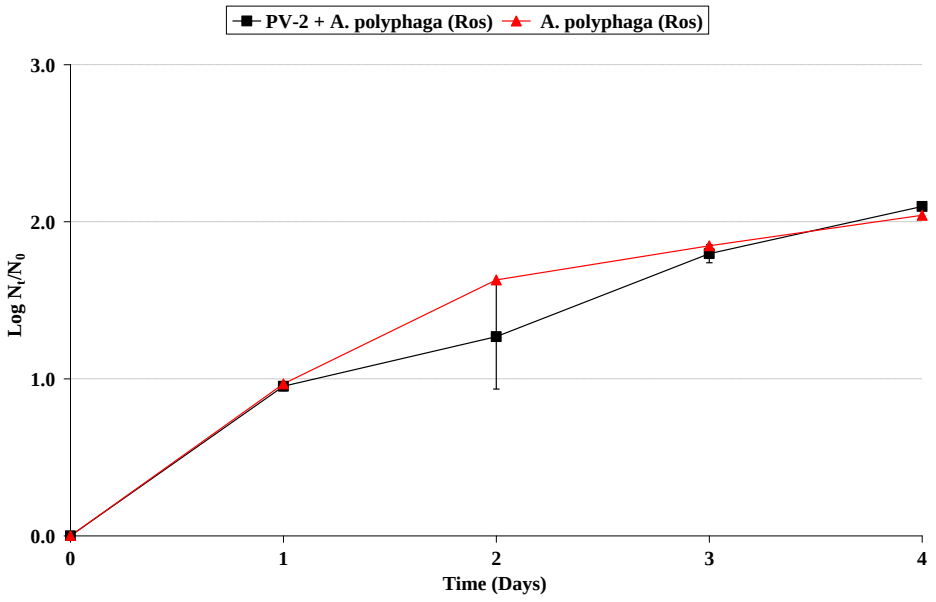


Figure 3.2. Growth of *A. polyphaga* (Ros) co-cultured with poliovirus type 2 (PV-2) ( ) and without PV-2 virus ( ) ($P \geq 0.8$), n=3.

The titre of coxsackievirus B5 (CVB5F) when co-cultured with *A. polyphaga* (Ros) showed no difference from culture of virus alone (t test $P \geq 0.5$) (Figure 3.3) and the results are comparable to those seen with PV-2 virus cultured with *A. polyphaga* (Ros), as can be seen in Figure 3.1.

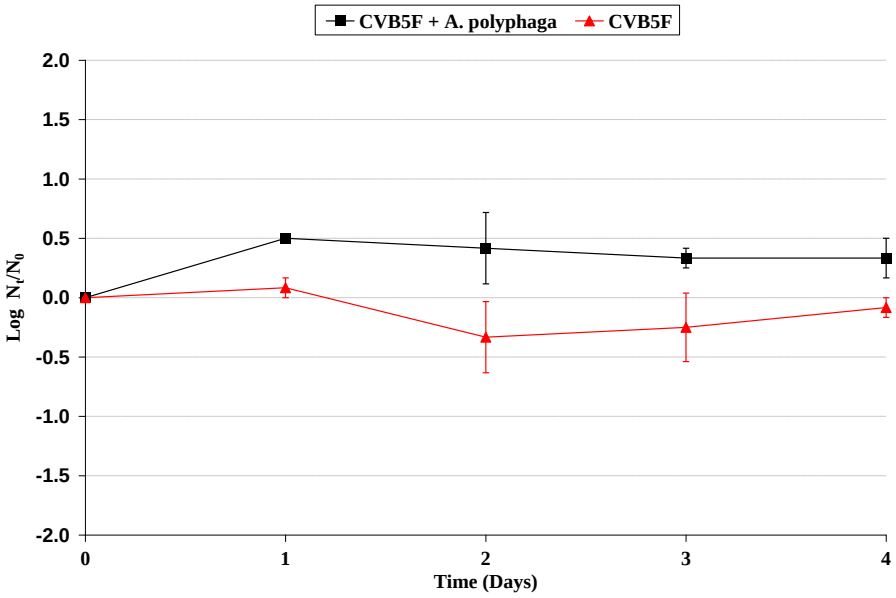


Figure 3.3. Titre of coxsackievirus B5 (CVB5F) either alone ( ) or co-cultured with *A. polyphaga* (Ros) ( ). There was no significant difference in the titre of CVB5F alone or when co-cultured with *A. polyphaga* (Ros) ($P \geq 0.5$), n=3.

The co-culture of CVB5F with A. *polyphaga* (Ros) cells had no effect on cell growth or viability compared to the control cells without virus (t test $P \geq 0.2$) (Figure 3.4).

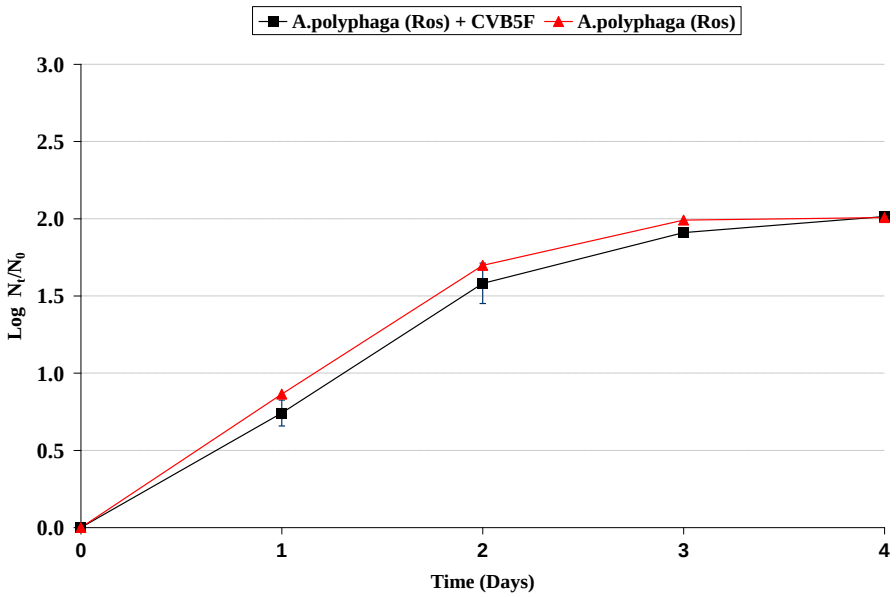


Figure 3.4. Growth of *A. polyphaga* (Ros) co-cultured with coxsackievirus B5 (CVB5F) ( ) and without CVB5F virus ( ) ($P \geq 0.2$), n=3.

Figure 3.5 shows the results of an experiment to determine if incubation of CVB3N with amoeba had any effect on virus titre, (t test $P \geq 0.4$), and showed that *A. castellanii* replicated at the same rate as the virus-free control of *A. castellanii* cells and the (t test $P \geq 0.4$) (Figure 3.6).

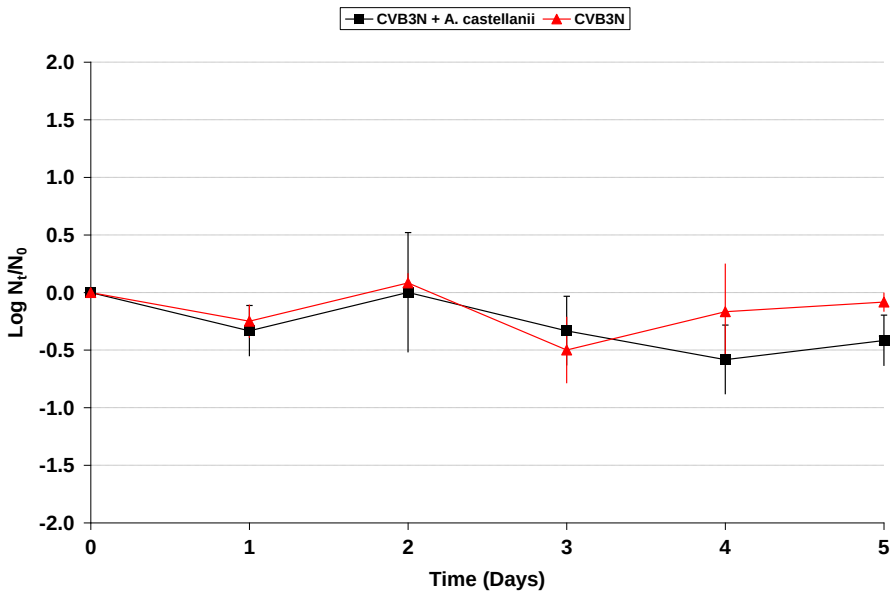


Figure 3.5. Titre of coxsackievirus B3 Nancy (CVB3N) either alone ( ) or co-cultured with *A. castellanii* (ATCC50370) ( ). There was no significant difference in the titre of CVB3N alone or when co-cultured with *A. castellanii* ($P \geq 0.4$), n=3.

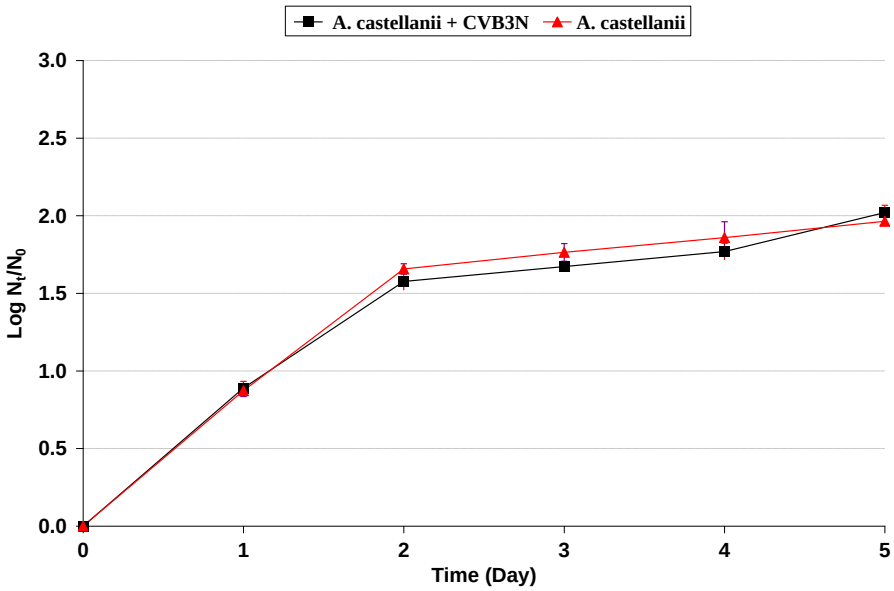


Figure 3.6 Growth of *A. castellanii* (ATCC50370) co-cultured with CVB3N ( ) and without CVB3N virus ( ) ($P \geq 0.4$), n=3.

Figure 3.7 shows the results of the experiment to determine if incubation of CVB5 with amoeba had any effect on the virus titre. There was no change in the viral titre when compared with the control viral culture without *A. castellanii* (ATCC50370) in #6 basal medium giving a (t test $P \geq 0.4$).

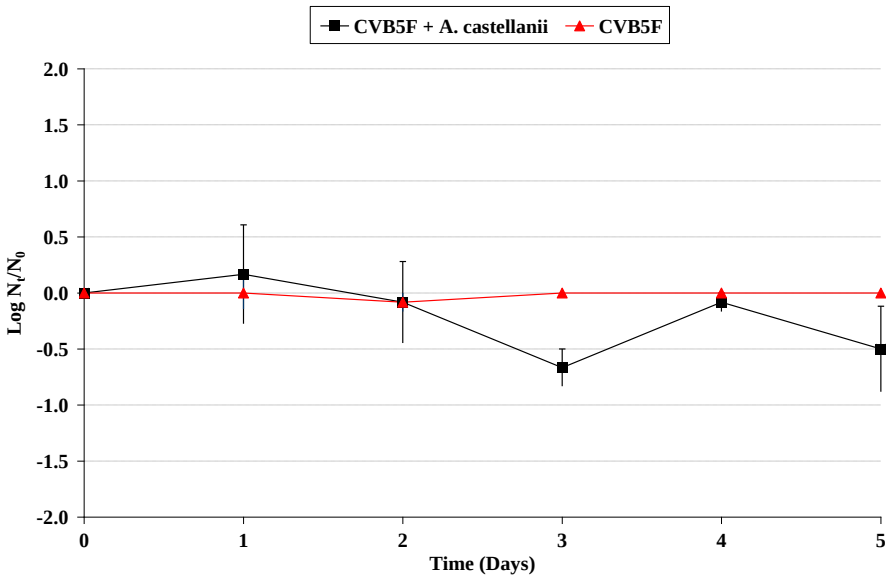


Figure 3.7. Titre of coxsackievirus B5 Faulkner (CVB5F) either alone (▲) or co-cultured with *A. castellanii* (ATCC50370) (■). There was no significant difference in the titre of CVB5F alone or when co-cultured with *A. castellanii* ($P \geq 0.4$), n=3.

In addition, the presence of CVB5F did not have an effect on the number of *A. castellanii* (ATCC50370) cells, (t test $P \geq 0.3$), which grew and replicated healthily as compared with the control without virus (Figure 3.8).

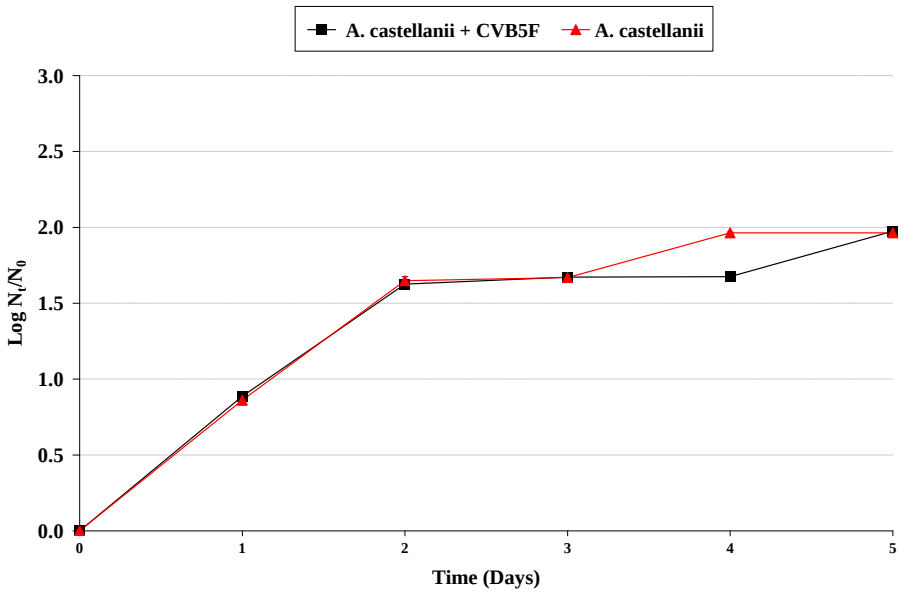


Figure 3.8. Growth of *A. castellanii* (ATCC50370) co-cultured with coxsackievirus B5 Faulkner (CVB5F) (■) and without CVB5F (▲) ($P \geq 0.3$), n=3.

Figure 3.9 shows the results of experiments to determine if incubation of PV-2 with *A. castellanii* (ATCC50370) had any effect on viral titre. There was no significant change when compared with the virus-only control, (t test $P \geq 0.1$). In addition *A. castellanii* cell growth was unaffected by the presence of the virus (t test $P \geq 0.2$) (Figure 3.10).

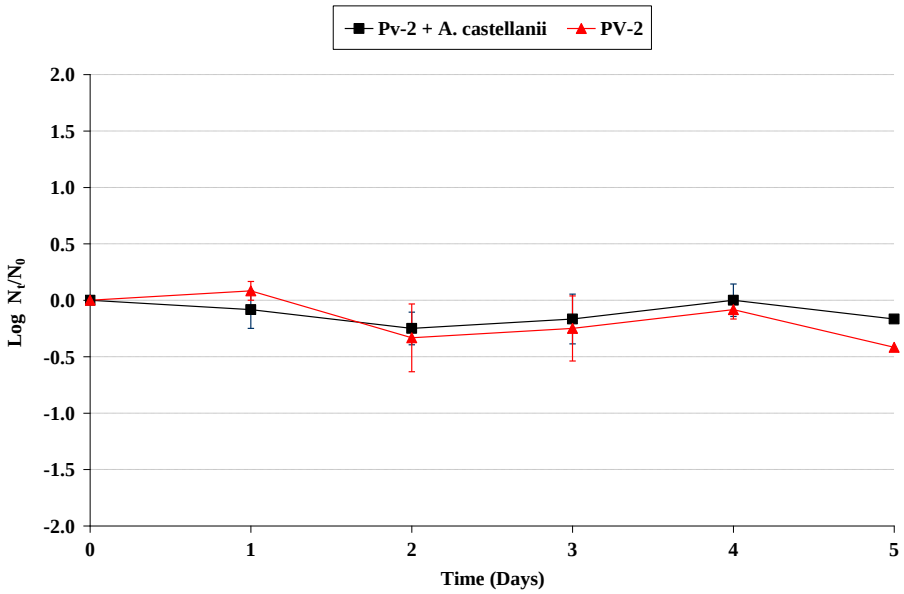


Figure 3.9. Titre of poliovirus type 2 (PV-2) either alone ( ) or co-cultured with *A. castellanii* (ATCC50370) ( ). There was no significant difference in the titre of PV-2 alone or when co-cultured with *A. castellanii* ($P \geq 0.1$), n=3.

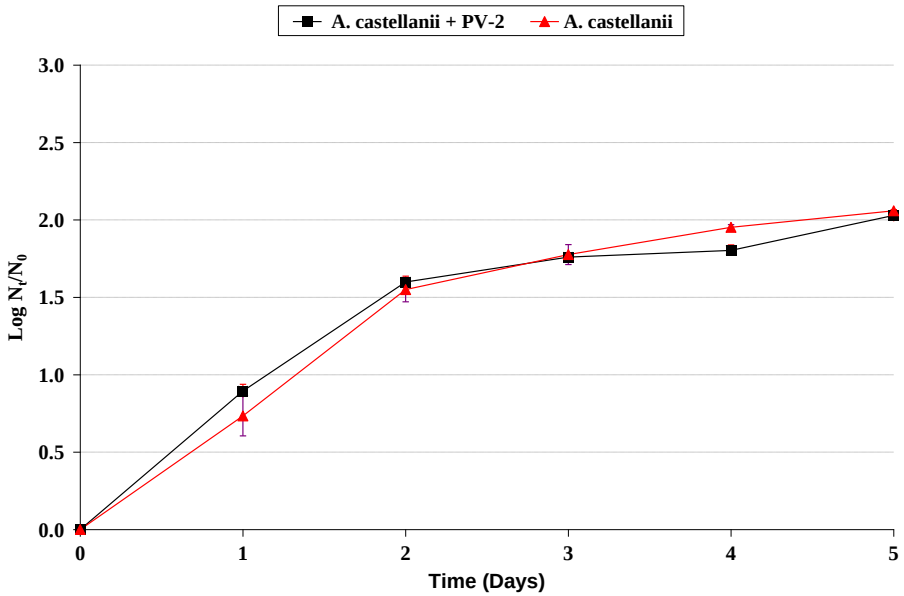


Figure 3.10. Growth of *A. castellanii* (ATCC50370) co-cultured with poliovirus type 2 (PV-2) and without *A. castellanii* ($P \geq 0.2$), n=3.

3.1.4 Determination of viral uptake by A. *castellanii*

Using the method of Mattana *et al.*, (2006), experiments were performed to assess the degree of viral internalization within *A. castellanii.* Following 24h or 48h co-culture *A. castellanii* was stained with strain specific antibodies to detect viral particles using indirect immunofluorescence.

3.1.4.1 Indirect immunofluorescence detection of viruses

Indirect immunofluorescence sensitivity tests were performed to determine the optimum dilution of antibody on mammalian cells infected with virus (CVB3N, CVB5F, PV-2 and RV Wa) (Table 3.1). The optimum dilution for detecting CVB3N particles with antibody was 1:3200, for CVB5F it was 1:400, for PV-2, 1:400 and for RV Wa, 1:40.

Table 3.2. Optimum dilution of mouse anti-virus antibody for detecting viral particles in suspension

Antibody	Virus	Optimum dilution
Mouse anti-coxsackievirus B blend	CVB3N	1:3200
Mouse anti-coxsackievirus B blend	CVB5F	1:400
Mouse anti-poliovirus 2 monoclonal antibody	PV-2	1:400
Mouse anti-rotavirus monoclonal antibody	RV Wa	1:40

To confirm that the indirect immunofluorescence (IF) assay could detect internalised virus, HEp-2 cells were infected with CVB3N, incubated for 24 hr, fixed in methanol and tested. As can be seen from Figure 3.11 the virus was clearly detected in infected cells and there was no IF seen with non-infected control mammalian cells (Fig 3.12). However, using this same method no fluorescence was seen in *A. castellanii* following co-culture with CVB3N as can be seen in Figure 3.13A which is similar to non-infected *A. castellanii* (Figure 3.14A).

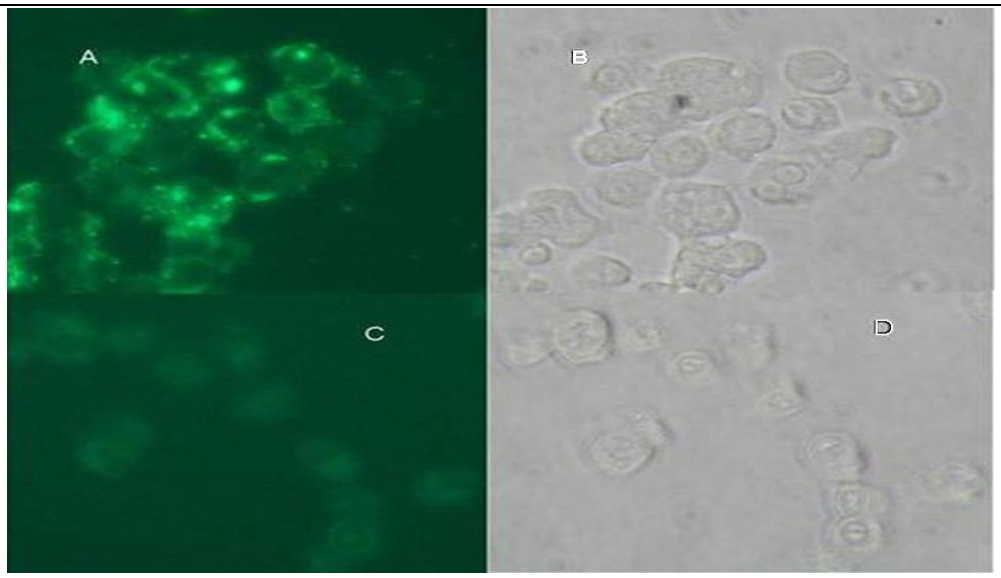


Figure 3.11. Indirect immunofluorescence of (positive control) A) CVB3N infected HEp-2 cells after 24 hours culture stained with mouse anti-coxsackievirus (B blend) B) Light field of A. C) CVB3N infected HEp-2 cells stained with secondary antibody and D) Light field of cells showing C. (x400).

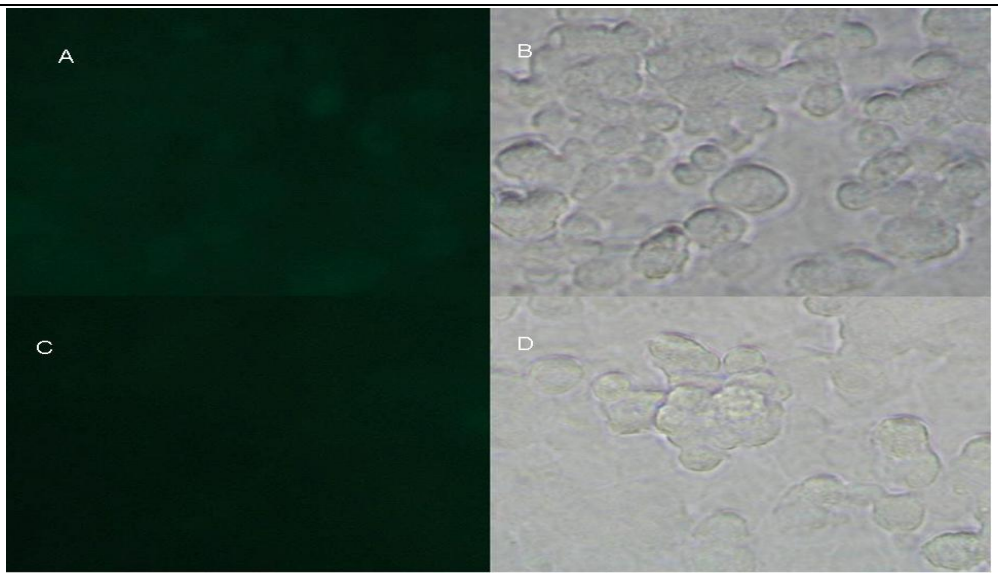


Figure 3.12. Indirect immunofluorescence of (negative control) A) Non-infected HEp-2 cells stained with anti-coxsackievirus (B blend) primary antibody and anti-mouse immunoglobulin G (IgG) FITC conjugate, whole molecule secondary antibody, B) Light field of cells showing A.; C) Non-infected HEp-2 cells stained with secondary antibody; D) Light picture of cells showing C. (x400).

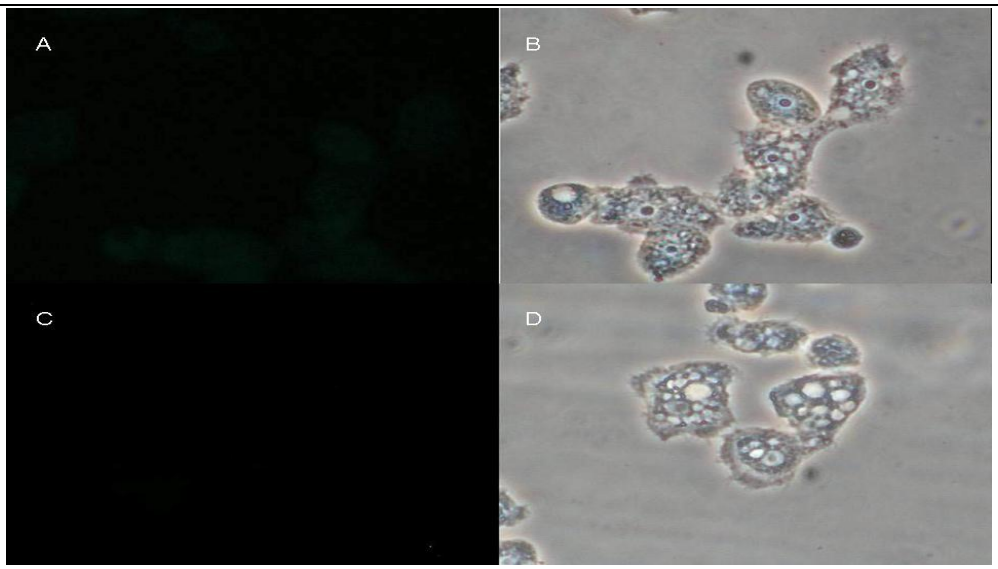


Figure 3.13. Indirect immunofluorescence of A) *A. castellanii* (ATCC50370) co-cultured with CVB3N stained with mouse anti-coxsackievirus (B blend) B) Light field of A. C) *A. castellanii* co-cultured with CVB3N stained with secondary antibody alone D) Light field of C. (x400).

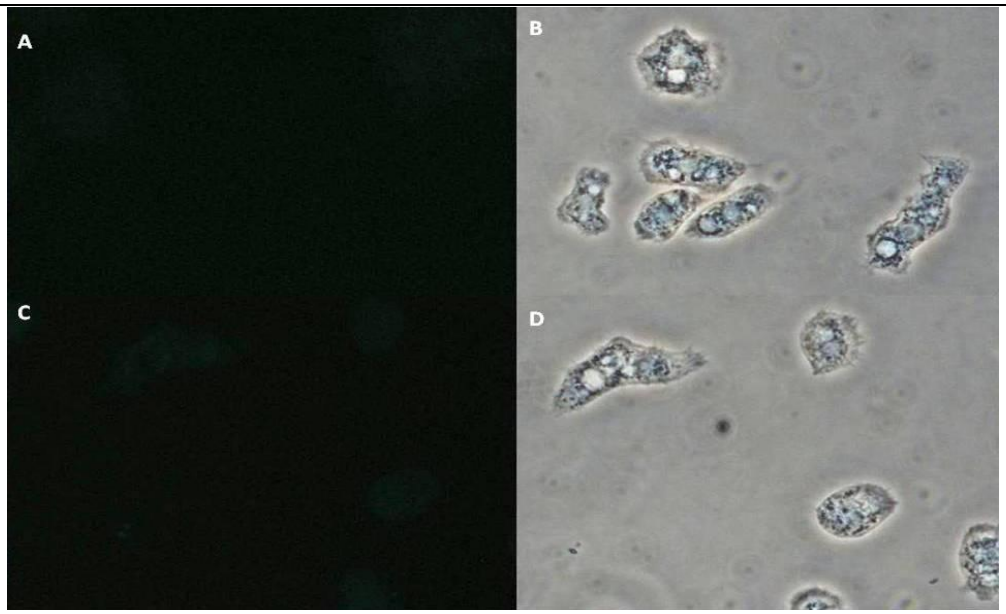


Figure 3.14. Indirect immunofluorescence of (negative control) A) *A. castellanii* (ATCC50370) alone stained with mouse anti-coxsackievirus (B blend). B) Light picture of A. C) *A. castellanii* stained with secondary antibody and D) Light field of C. (x400).

A similar series of experiments were performed using coxsackievirus B5 (CVB5F) (Figures 3.15-3.18) and, again, although the method was clearly able to detect viral particles in Hep-2 infected cells (Figure 3.15A) none were seen in *A. castellanii* co-cultured with the virus (Figure 3.17A).

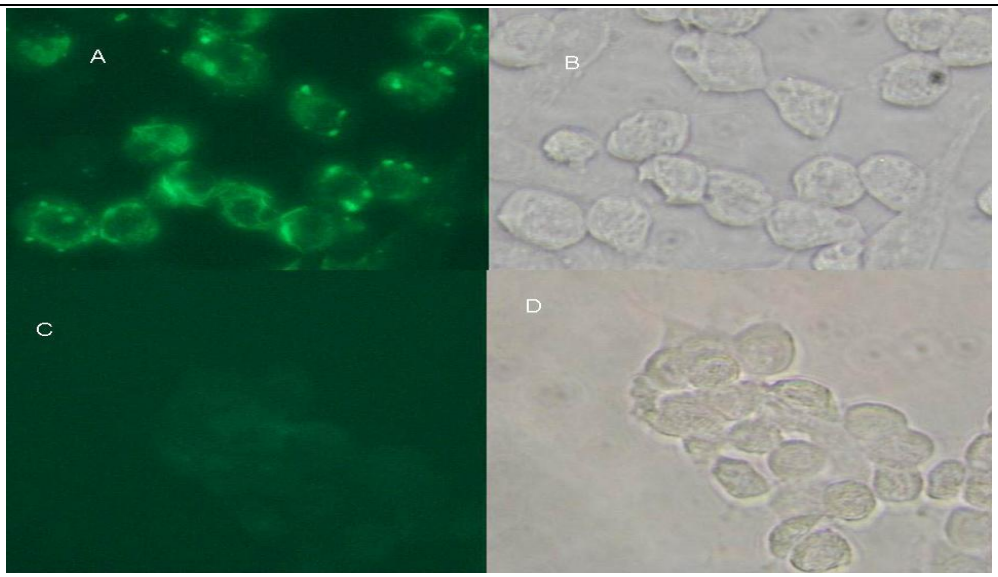


Figure 3.15. Indirect immunofluorescence of (positive control) A) CVB5F infected HEp-2 after 24 hours culture stained with mouse anti-coxsackievirus (B blend). B) Light field of A. C) CVB5F infected HEp-2 stained with secondary antibody D) Light field of C. (x400).

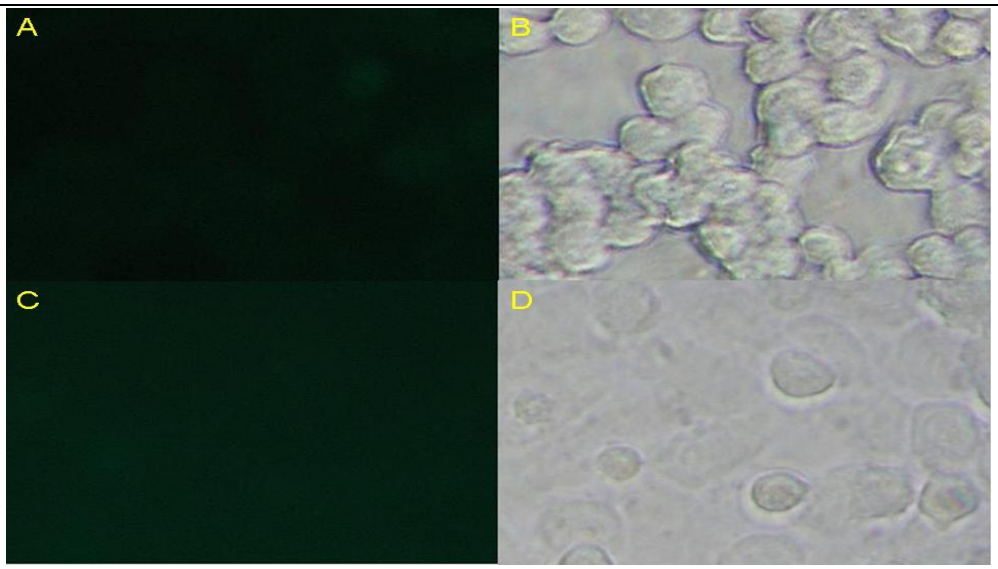


Figure 3.16. Indirect immunofluorescence of (negative control) A) Non-infected HEp-2 cells stained with mouse anti-coxsackievirus antibody (B blend). B) Light field of A. C) Non-infected HEp-2 cells stained with secondary antibody D) Light field of C. (x400).

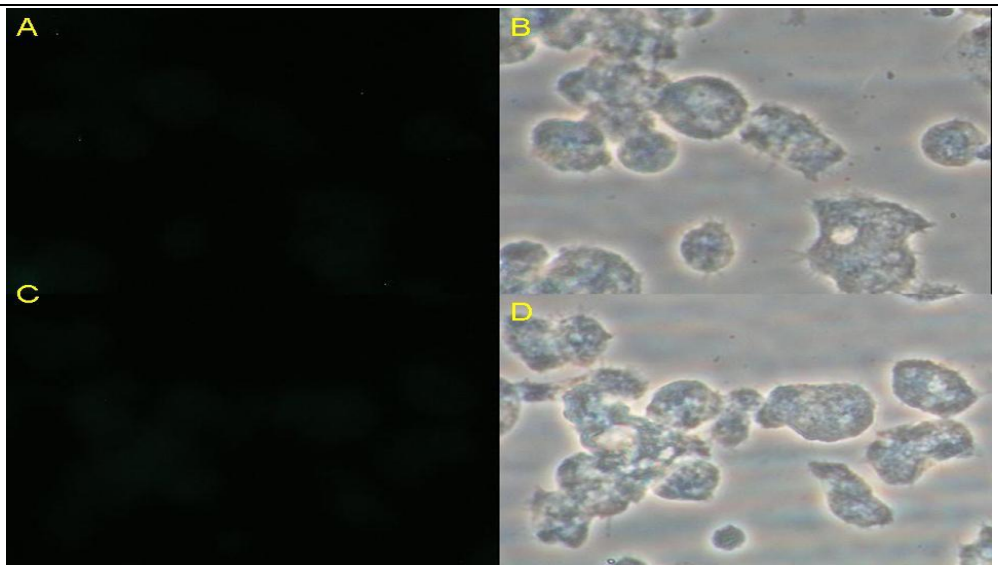


Figure 3.17. Indirect immunofluorescence of A) *A. castellanii* (ATCC50370) co-cultured with CVB5F stained with mouse anti-coxsackievirus antibody (B blend). B) Light field of A. C) *A. castellanii* (ATCC50370) co-cultured with CVB5F stained with secondary antibody D) Light field of C. (x400).

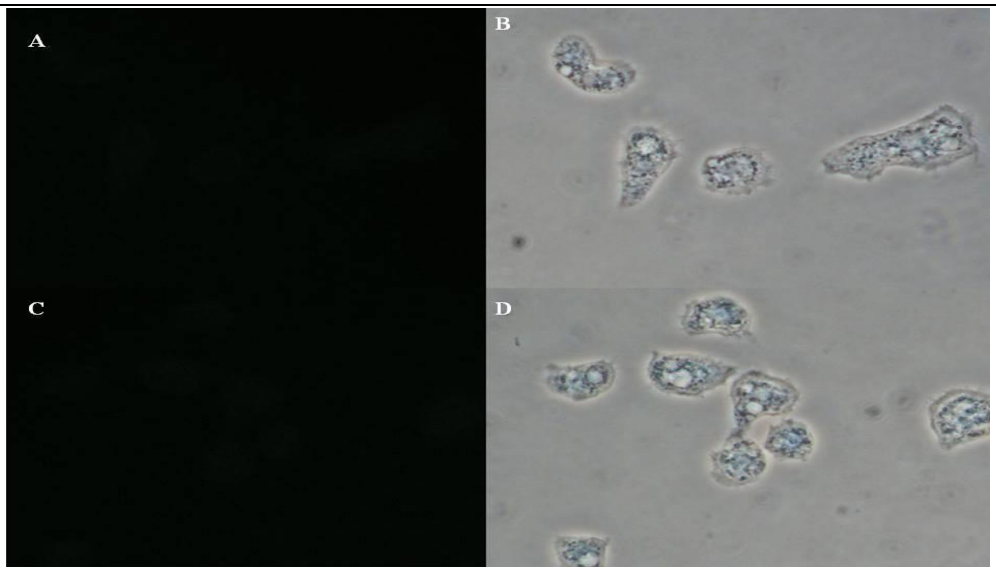


Figure 3.18. Indirect immunofluorescence of (negative control) A) *A. castellanii* (ATCC50370) alone stained with mouse anti-coxsackievirus (B blend). B) Light picture of A. C) *A. castellanii* stained with secondary antibody and D) Light field of C. (x400).

In a further series of experiments using poliovirus (PV-2), although the virally infected Hep-2 cells were strongly positive (Figure 3.19A) no viral uptake was observed with *A. castellanii* co-cultured with the virus (Figure 3.21A).

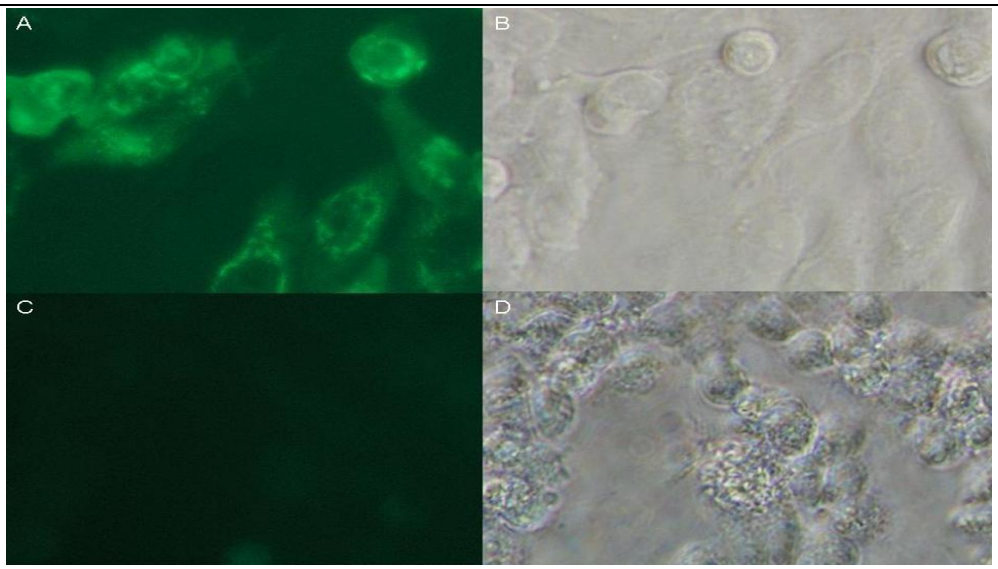


Figure 3.19. Indirect Immunofluorescence of (positive control) A) PV-2 infected HEp-2 stained with mouse anti-poliovirus 2 monoclonal antibody B) Light field of A. C) PV-2 infected HEp-2 with secondary antibody and D) Light field of C. (x400).

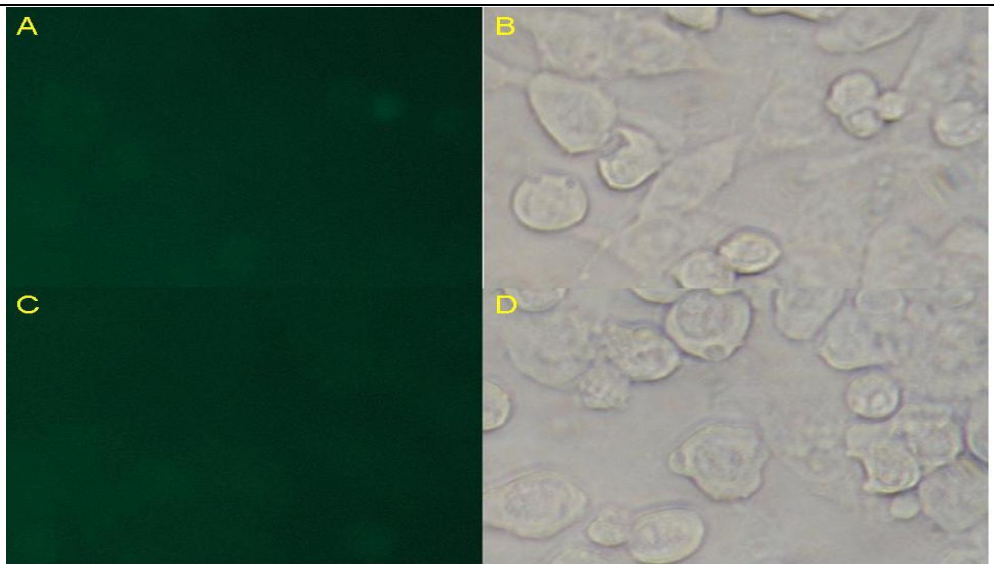


Figure 3.20. Indirect Immunofluorescence of (negative control) A) Non-infected HEp-2 cells stained with mouse anti-poliovirus 2 monoclonal. B) Light field of A. C) Non-infected HEp-2 cells stained with secondary antibody. D) Light field of C. (x400).

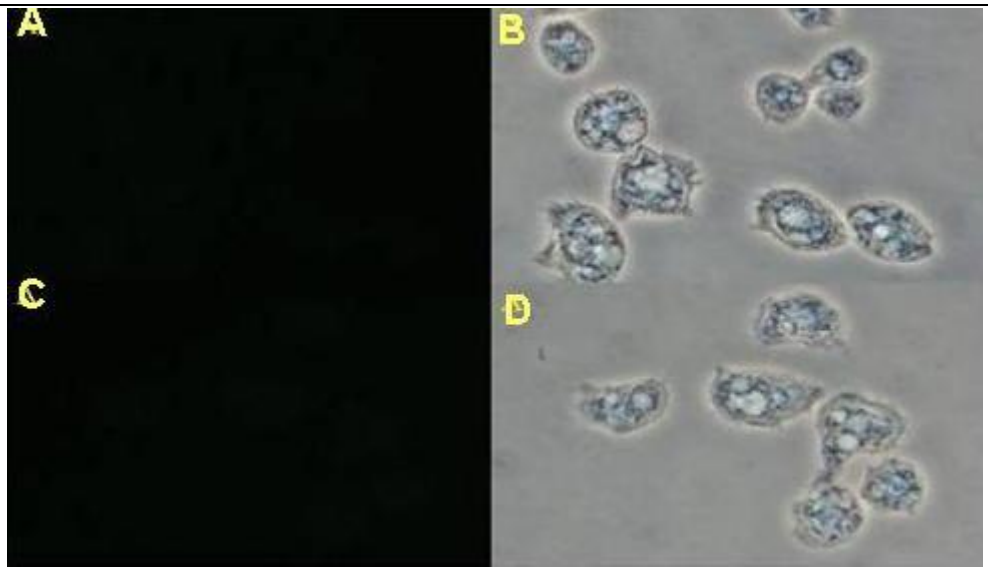


Figure 3.21. Indirect immunofluorescence picture of A) *A. castellanii* (ATCC50370) co-cultured with PV-2 stained with mouse anti-poliovirus 2 monoclonal antibody. B) Light field of A. C) *A. castellanii* (ATCC50370) co-cultured with PV-2 stained with secondary antibody. D) Light field of C. (x400).

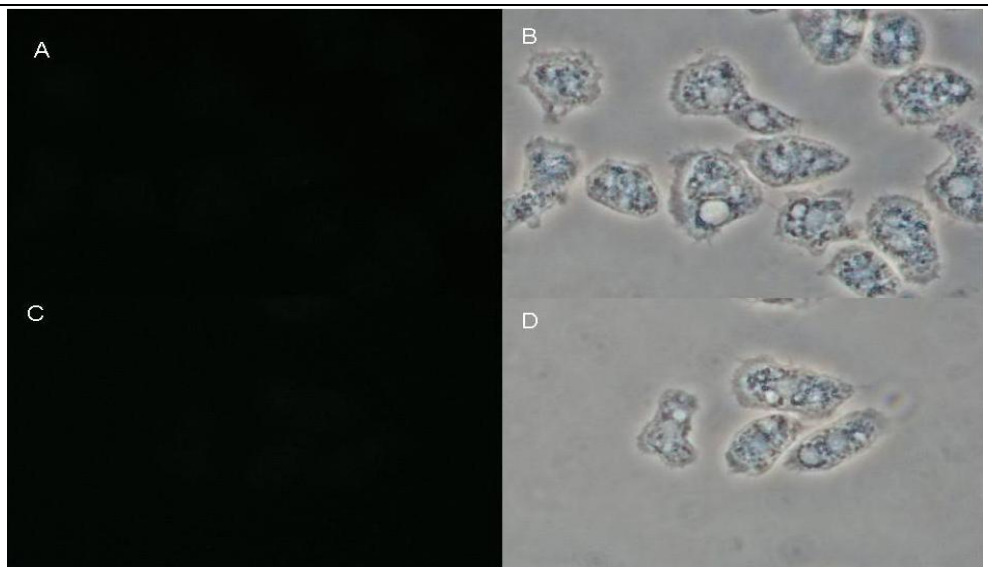


Figure 3.22. Indirect immunofluorescence of (negative control) A) *A. castellanii* (ATCC50370) stained with mouse anti-poliovirus 2 monoclonal antibody. B) Light field of A. C) *A. castellanii* stained with secondary antibody and D) Light field of C. (x400).

To determine whether rotavirus (RV Wa) could be detected by this method within cells, MA104 cells were directly infected with the virus and subjected to the IF method as above using a mouse anti-rota virus antiserum. As can be seen in Figure 3.23A, the virus was readily detected, although, as with the experiments described above, *A. castellanii* co-cultured for 24h with the virus did not show any fluorescence (Figure 3.25A).

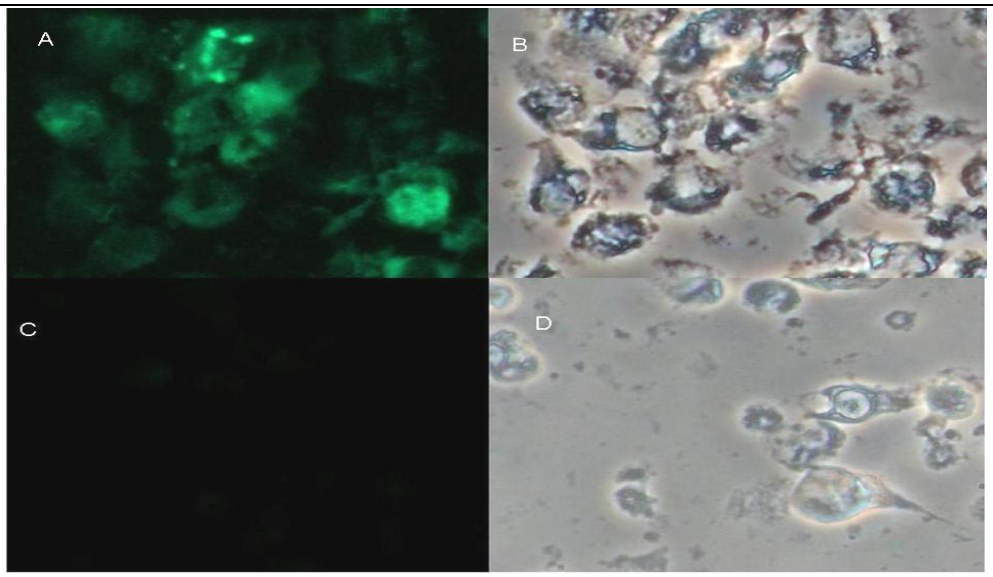


Figure 3.23. Immunofluorescence of (positive control) A) Rotavirus Wa infected MA104 cells stained with mouse anti-rotavirus monoclonal primary antibody. B) Light field of A. C) MA104 cells infected with rotavirus Wa stained with secondary antibody. D) Light field of C. (x400).

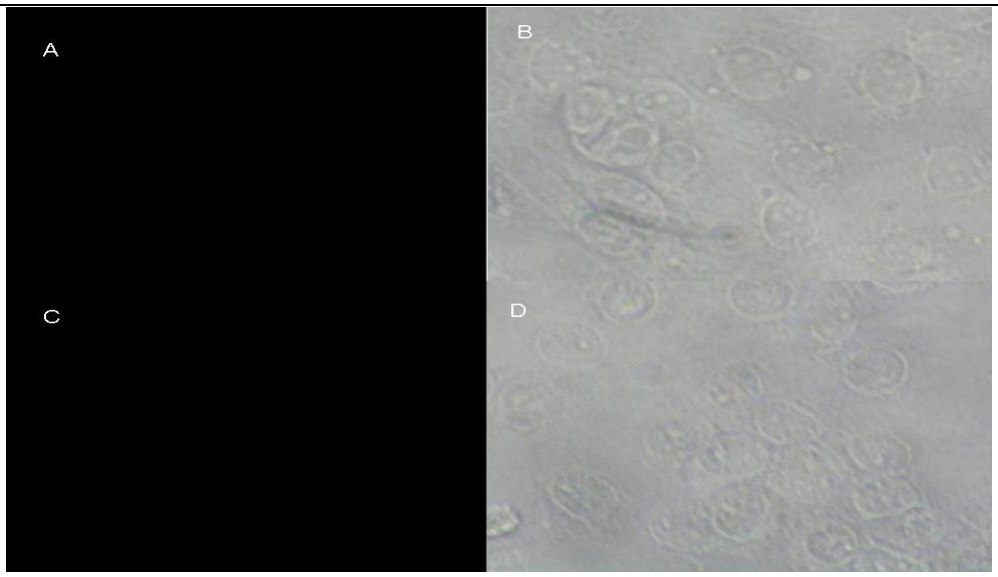


Figure 3.24. Immunofluorescence of (negative control) A) non-infected MA104 cells stained with mouse anti-rotavirus monoclonal primary antibody. B) Light field of A. C) non-infected MA104 cells stained with secondary antibody and D) Light field of C. (x400).

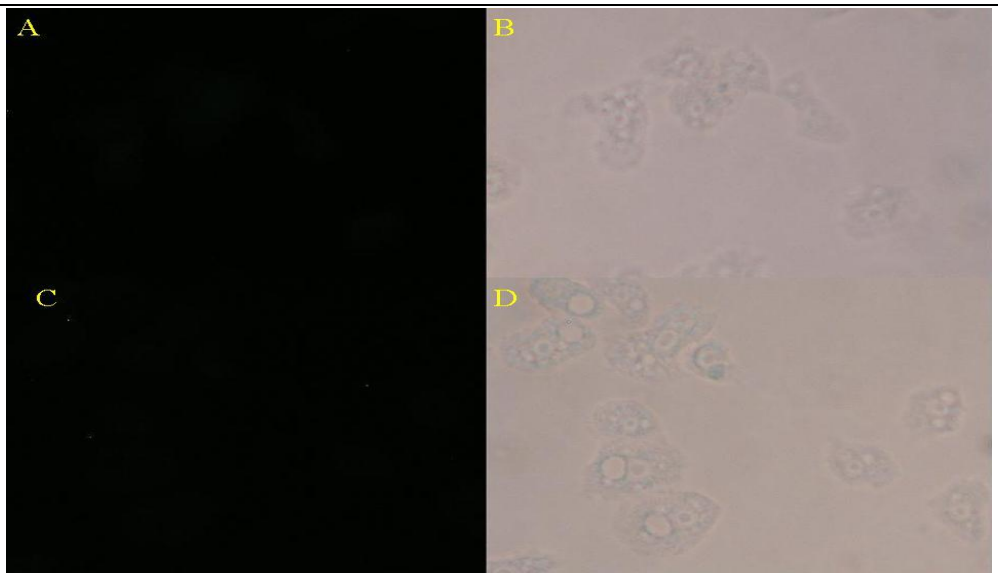


Figure 3.25. Indirect immunofluorescence of A) *A. castellanii* (ATCC50370) co-cultured with RV Wa stained with mouse anti-rotavirus monoclonal primary antibody. B) Light field of A. C) *A. castellanii* (ATCC50370) co-cultured with RV Wa stained with secondary antibody. D) Light field of C. (x400).

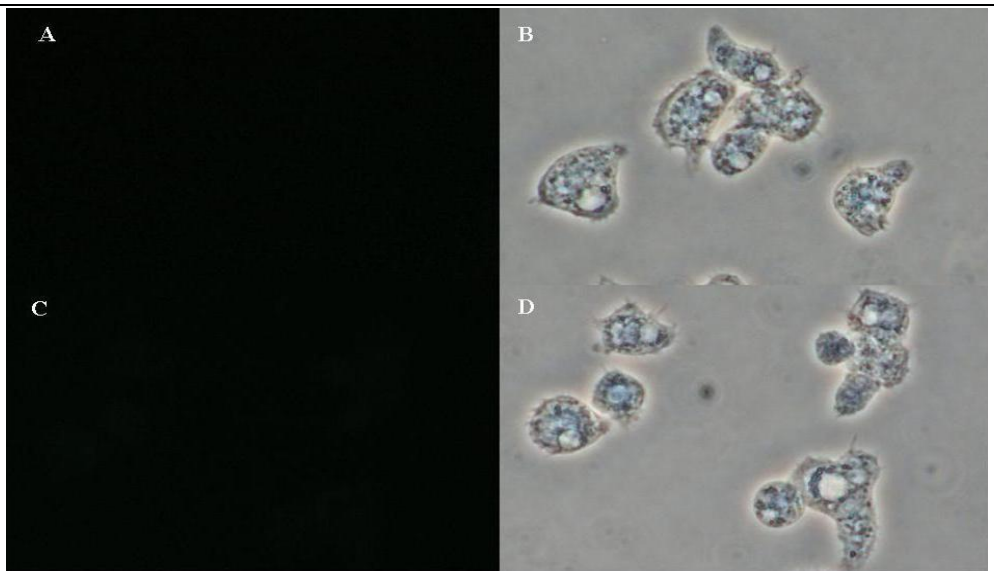


Figure 3.26. Indirect immunofluorescence of (negative control) A) *A. castellanii* (ATCC50370) stained with mouse anti-rotavirus monoclonal primary antibody. B) Light field of A. C) *A. castellanii* stained with secondary antibody. D) Light field of C. (x400).

3.1.5 Determination of the ability of *A. castellanii* trophozoites to adhere or internalise viral infected mammalian cells.

Although no direct uptake of viral particles by *A. castellanii* was observed (as can be seen from the data described above) another way in which these cells could act as carriers for viral particles is by internalising other cell types which are already virally infected. To test this hypothesis, CVB3N, CVB5F and PV-2 infected Hep-2 cells and

RV Wa infected MA104 cells were co-cultured (fed) with *A. castellanii* trophozoites. Following 18h co-culture mammalian cells were engulfed by the amoeba and it was possible to then detect viral particles by indirect immunofluorescence within *A. castellanii* (Figures 3.27 and 3.28). Although it was clearly possible to show uptake of CVB3N and RV Wa virally infected cells, no uptake of CVB5F and PV-2 infected HEp-2 cells was detected (data not shown).

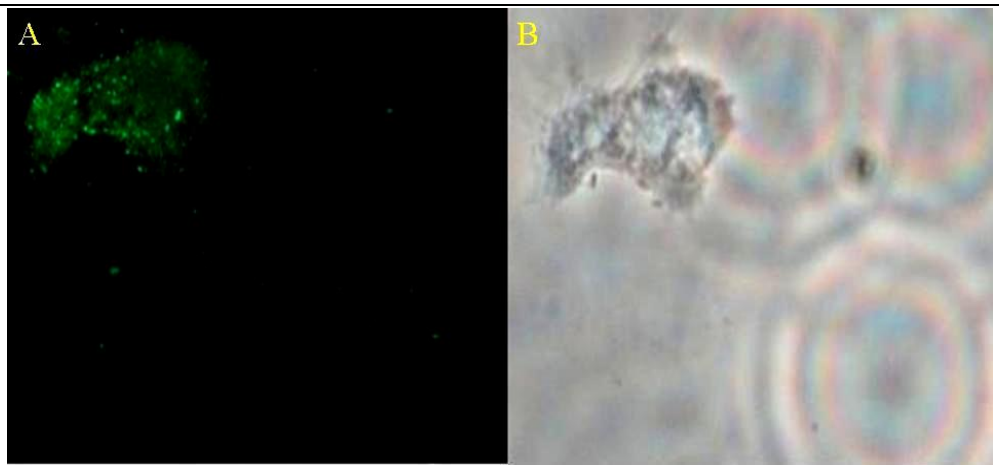


Figure 3.27. Indirect immunofluorescence of A) *A. castellanii* (ATCC50370) fed on HEp-2 cells infected with CVB3N stained with mouse anti-coxsackievirus (B blend) primary antibody and B) Light field of A. (x400).

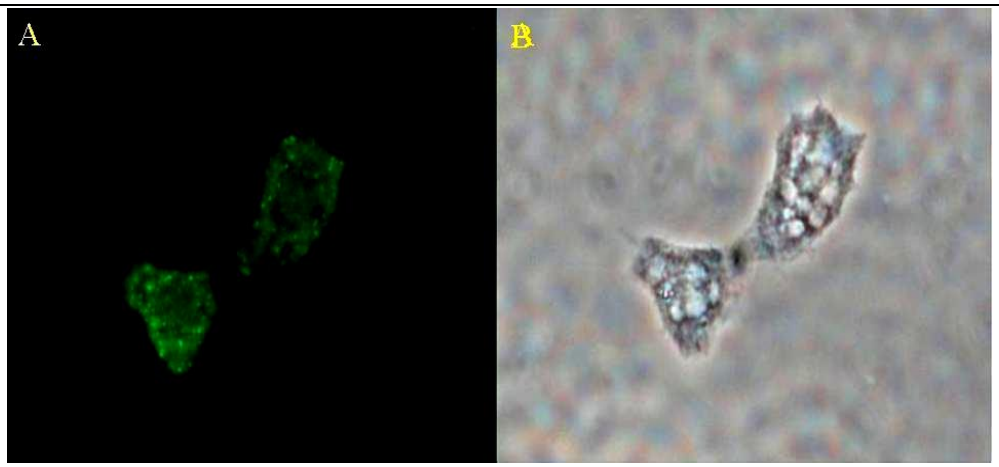


Figure 3.28. A) *A. castellanii* (ATCC50370) fed on MA104 cells infected with RV Wa stained with mouse anti-rotavirus monoclonal primary antibody and B) Light field of A. (x400).

3.1.6 Assessment of viral internalisation following encystment of *A. castellanii*

A. castellanii was co-cultured with the viral preparations described earlier (i.e. CVB3N, CVB5F, PV-2 and RV Wa) by the protocol of Mattana *et al.* (2006) and the trophozoites encysted in PBS or in the encystment medium of Neff, as described in Chapter 2 section 2.2.7, followed by excystment in #6 basal medium. Cell culture was used to test for the presence of infective viral particles, although no CPE was observed (data not shown). Further tests (see below) were carried out to confirm these observations.

3.1.7 Determination of viral infectivity on Mammalian cells

A sample of the co-culture suspension of virus / *A. castellanii* was taken to assess viral infectivity (CVB3N, CVB5F and PV-2) on Hep-2 cells or for RV Wa on MA104 cells. The co-culture sample was frozen/thawed 3-6 times but these did not produce a detectable cytopathic effect on inoculated mammalian cells following 7 days incubation at 37°C in a CO_2 a humid incubator. CPE effect was observed of co-cultures of *A. castellanii* before encystment with CVB3N and RV Wa infected mammalian cells. While the other co-culture experiment results did not show CPE (data not shown).

3.1.8 Encystment of *A. castellanii* fed on viral infected Mammalian cells

Although in the experimental system shown above (Figures 3.27 and 3.28) CVB3N and RV Wa viruses were detected by indirect immunofluorescence associated with *Acanthamoeba* (confirmed by RT-PCR – see section 3.2), following encystment no detectable cytopathic effect was observed on mammalian cells and no viral RNA was detected using RT-PCR (for results of these experiments see section 3.2).

3.2 RT-PCR results

3.2.1 Preparation of purified viral RNA

RNA concentrations were quantified using a NanoDrop™ 2000 spectrophotometer (Thermo Scientific NanoDrop™ 2000, V3.3.0) (see Methods Chapter 2). Table 3.3 shows the concentrations of RNA obtained from each viral or viral/*Acanthamoeba* preparation.

Table 3.3. Concentrations of total RNA obtained from the viral or viral/*Acanthamoeba* co-cultures (1 x 10^6 $TCID_{50}$/ml) using the NanoDrop™ 2000.

Sample	RNA concentration (ng/µl)
CVB3N	63
CVB5F	52
PV-2	93.9
RV Wa	94.8
A. castellanii	73
A. castellanii co-cultured with coxsackievirus B3 Nancy	70
A. castellanii co-cultured with coxsackievirus B5 Faulkner	46
A. castellanii co-cultured with poliovirus type 2	75
A. castellanii co-cultured with rotavirus Wa	92

3.2.2 RT-PCR assessment of viral RNA in *Acanthamoeba* co-culture experiments (Sensitivity test, viral/*Acanthamoeba* co-culture and virally infected mammalian cells/Acanthomeaba co-culture).

3.2.2.1 CVB3N

In order to determine the minimum amount of viral RNA that could be detected using the RT-PCR system described in Chapter 2, a sensitivity test was performed. CVB3N virus stock was prepared as described in Chapter 2 and from the stock concentration; a sample of 10^7 $TCID_{50}$/ml was prepared which was serially diluted, incubated with the CVB3N specific PCR primers for RT-PCR before being resolved on an agarose gel as described earlier (Chapter 2). In the example shown for in Figure 3.29 CVB3N (which was typical of the other viruses tested) the lowest concentration of viral RNA which could be detected was 10^2 $TCID_{50}$/ml (Figure 3.29). From this it was possible to calculate (140µl taken for extraction from virus stock 1000µl) that RT-PCR was able to detect 14 infective viral units per sample for CVB3N (see Chapter 2).

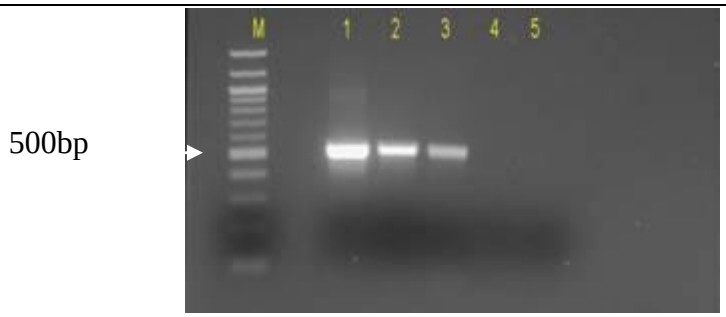


Figure 3.29. RT-PCR Sensitivity test for detection of CVB3N viral RNA from CVB3N infected HEp-2 cells. Serially dilutions of viral suspension following amplification with the CVB3N specific PCR probe which detects an 521 bp band. Lane 1) 10^7 $TCID_{50}$/ml; 2) 10^5 $TCID_{50}$/ml; 3) 10^2 $TCID_{50}$/ml; 4) 10 $TCID_{50}$/ml and 5) CVB3N primers alone as the RT-PCR negative control. M = DNA ladder 100 bp.

The negative indirect immunofluorescence results obtained following the co-culture of *A. castellanii* with CVB3N (Figure 3.13) were confirmed by RT-PCR (Figure 3.30) which included both a positive [stock CVB3N] and negative [primers alone] controls (Figure 3.30, Lanes 2 & 4).

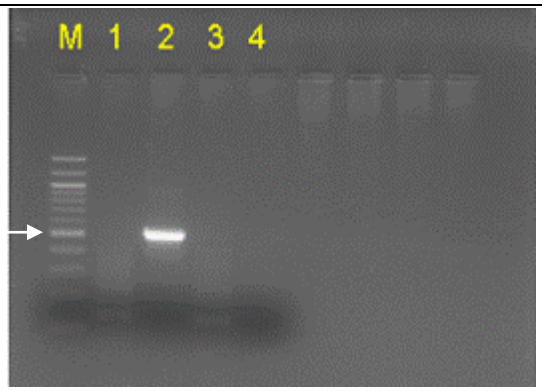


Figure 3.30. Assessment of the presence of CVB3N sequences in cell extracts following co-culture of CVB3N with *A. castellanii* by RT-PCR amplification. Lane 1) Co-culture of CVB3N with *A. castellanii* (ATCC50370); 2) positive control CVB3N stock [521 bp]; 3) *A. castellanii* alone and 4) primers alone negative control. M = 100bp DNA ladder.

Co-cultures of *A. castellanii* fed on HEp-2 cells infected with CVB3N were positive using IF (Figure 3.27) and this was confirmed by RT-PCR (Figure 3.31) this shows an association of the viral infected HEp-2 cells with *A. castellanii* which confirms virus uptake.

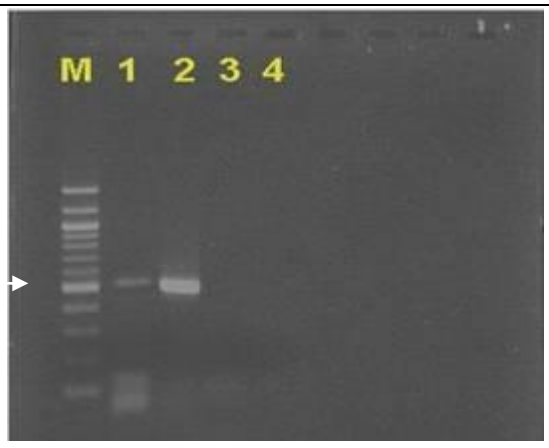


Figure 3.31. Assessment of the presence of CVB3N sequences in cell extracts following co-culture of *A. castellanii* with Hep-2 cells infected with CVB3N by RT-PCR. 1) *A. castellanii* (ATCC50370) fed on Hep-2 cells infected with CVB3N; 2) positive control CVB3N stock [521bp]; 3) *A. castellanii* alone 4) primers alone negative control. M) 100 bp DNA ladder.

3.2.2.2 CVB5F

The sensitivity test for the presence of CVB5F was carried out as with CVB3N as above. As can be seen from Figure 3.32 the lowest concentration that could be detected was 10^3 $TCID_{50}$/ml.

The negative indirect immunofluorescence results obtained following the co-culture of *A. castellanii* with CVB5F (Figure 3.17) were confirmed by RT-PCR (Figure 3.33) which included both a positive [stock CVB5F] and negative [primers alone] controls (Figure 3.33, Lanes 2 & 4). The co-culture experiments with *A. castellanii* fed with Hep-2 cells infected with CVB5F also proved negative using RT-PCR (data not shown).

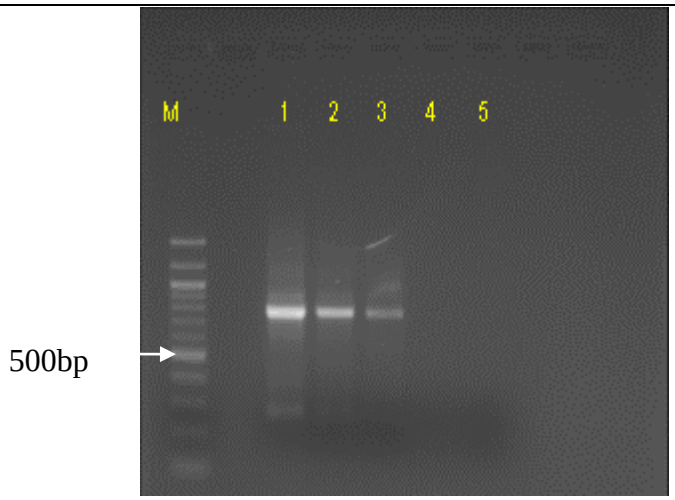


Figure 3.32. RT-PCR Sensitivity test for detection of CVB5F viral RNA from CVB5F infected HEp-2 cells. Serially dilutions of viral suspension following amplification with the CVB5F specific PCR probe which detects an 760 bp band. Lane 1) 10^7 TCID50/ml; 2) 10^5 TCID50/ml; 3) 10^3 TCID50/ml; 4) 10^2 TCID50/ml and 5) CVB5F primers alone as the RT-PCR negative control. M = DNA ladder 100 bp.

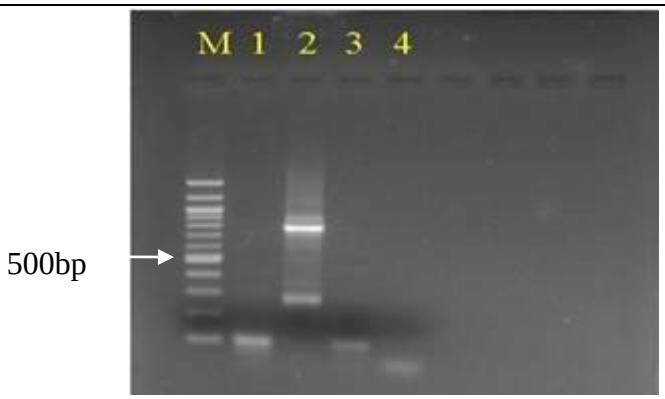


Figure 3.33. Assessment of the presence of CVB5F sequences in cell extracts following co-culture of CVB5F with *A. castellanii* by RT-PCR amplification. Lane 1) Co-culture of CVB5F with *A. castellanii* (ATCC50370); 2) positive control CVB5F stock [760 bp]; 3) *A. castellanii* alone and 4) primers alone negative control. M = 100bp DNA ladder.

4.2.3 PV-2

Before attempting to detect PV-2 RNA in the co-culture suspension using RT-PCR amplification, a sensitivity test of RT-PCR amplification was performed and this showed that the lowest concentration of PV-2 viral particles that could be detected in suspension was 10^3 $TCID_{50}$/ml (equivalent to 140 viral particles) as can be seen in Figure 3.34.

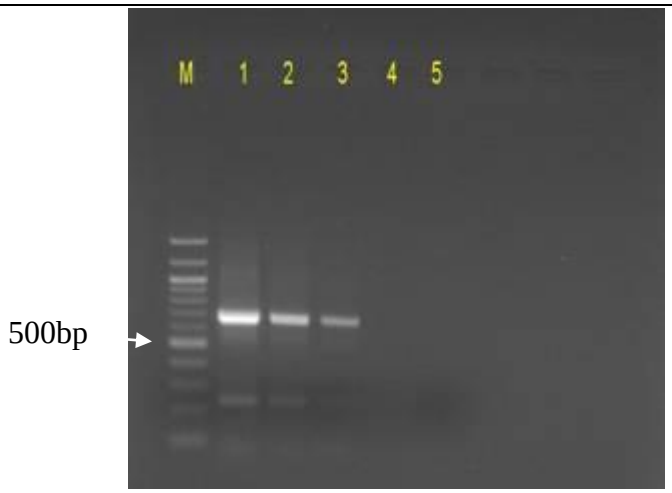


Figure 3.34. RT-PCR sensitivity test for detection of PV-2 viral RNA from PV-2 infected HEp-2 cells. Serially dilutions of viral suspension following amplification with the PV-2 specific PCR probe which detects an 650 bp band. Lane 1) 10^7 $TCID_{50}$/ml; 2) 10^5 $TCID_{50}$/ml; 3) 10^3 $TCID_{50}$/ml; 4) 10^2 $TCID_{50}$/ml and 5) PV-2 primers alone as the RT-PCR negative control. M = DNA ladder 100 bp.

The negative indirect immunofluorescence results obtained following the co-culture of *A. castellanii* with PV-2 (Figure 3.21) were confirmed by RT-PCR (Figure 3.35) which included both a positive (stock PV-2) and negative (primers alone) controls (Figure 3.35, Lanes 2 & 4). The co-culture experiments with *A. castellanii* fed with Hep-2 cells infected with PV-2 also proved negative using RT-PCR (data not shown).

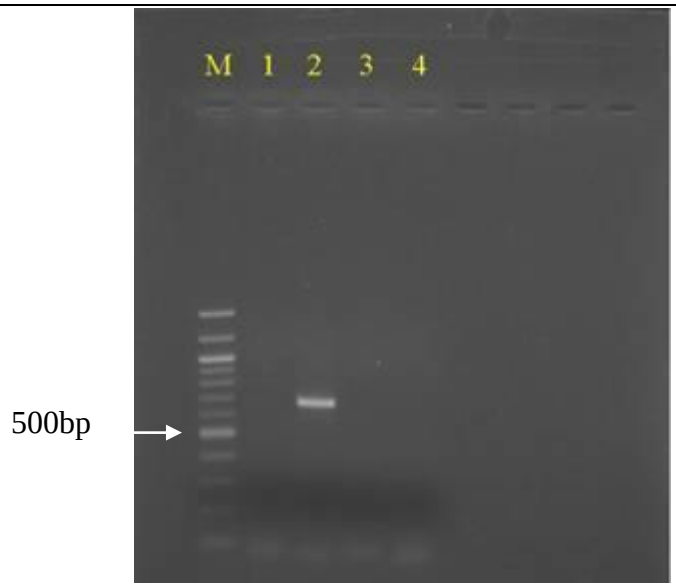


Figure 3.35. Assessment of the presence of PV-2 sequences in cell extracts following co-culture of PV-2 with *A. castellanii* by RT-PCR amplification. Lane 1) Co-culture of PV-2 with *A. castellanii* (ATCC50370); 2) positive control PV-2 stock [650 bp]; 3) *A. castellanii* alone and 4) primers alone negative control. M = 100bp DNA ladder.

4.2.4 RV Wa

The RT-PCR amplification sensitivity test was also performed for rotavirus Wa to determine the lowest concentration of viral particles that could be detected in the suspension. Samples used in this experiment were from an extraction of serially diluted concentrations from the viral stock that was obtained from ATCC using the QIAamp® Viral RNA Mini Kit. The results revealed that the minimum concentration that could be detected using RT-PCR amplification was 10^2 $TCID_{50}$/ml, which is equivalent to about 14 viral particles per 140 µl, as shown in Figure 3.36.

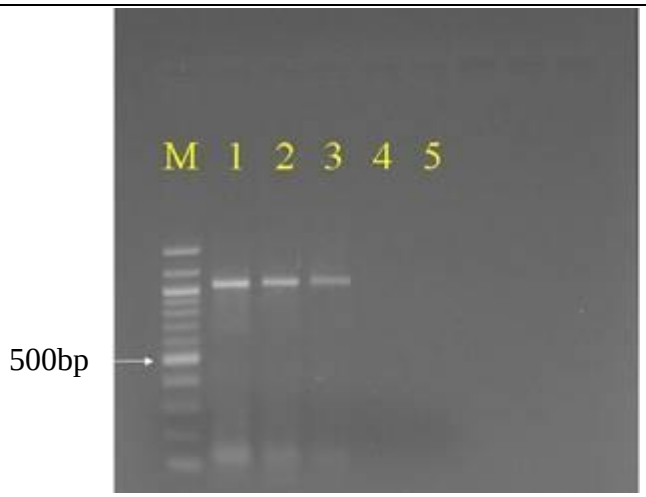


Figure 3.36. RT-PCR Sensitivity test for detection of RV Wa viral RNA from RV Wa infected MA104 cells. Serially dilutions of viral suspension following amplification with the RV Wa specific PCR probe which detects an 1062 bp band. Lane 1) 10^7 $TCID_{50}$/ml; 2) 10^5 $TCID_{50}$/ml; 3) 10^2 $TCID_{50}$/ml; 4) 10^1 $TCID_{50}$/ml and 5) RV Wa primers alone as the RT-PCR negative control. M = DNA ladder 100 bp.

The negative indirect immunofluorescence results obtained following the co-culture of *A. castellanii* with RV Wa (Figure 3.25) were confirmed by RT-PCR (Figure 3.37) which included both a positive (stock RV Wa) and negative (primers alone) controls (Figure 3.37, Lanes 2 & 4).

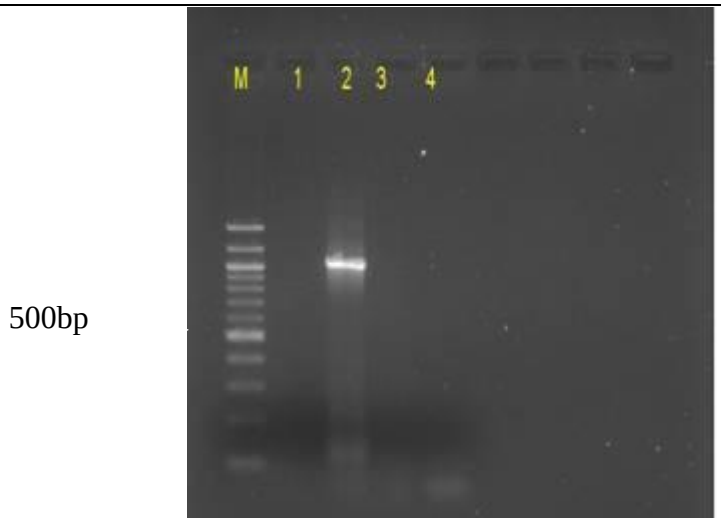


Figure 3.37. Assessment of the presence of RV Wa sequences in cell extracts following co-culture of RV Wa with *A. castellanii* by RT-PCR amplification. Lane 1) Co-culture of RV Wa with *A. castellanii* (ATCC50370); 2) positive control RV Wa stock [1062 bp]; 3) *A. castellanii* alone and 4) primers alone negative control. M = 100bp DNA ladder.

Co-cultures of *A. castellanii* fed on MA104 cells infected with RV Wa were positive using IF (Figure 3.28) and this was confirmed by RT-PCR (Figure 3.38) although this only confirms an association of the viral infected MA104 cells with *A. castellanii* and it is a confirmation of viral uptake.

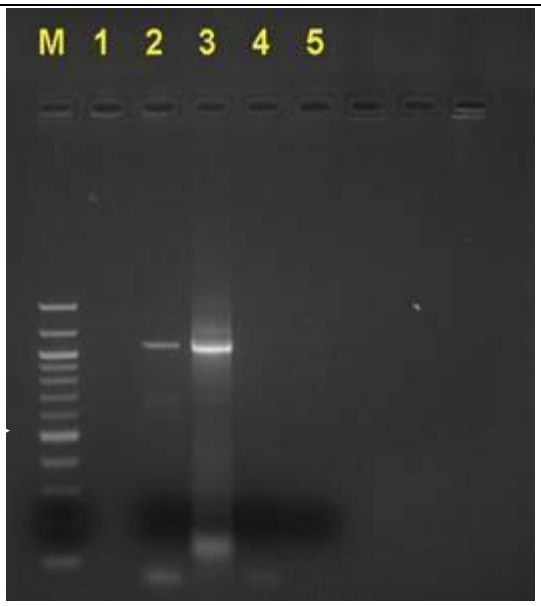


Figure 3.38. Assessment of the presence of RV Wa sequences in cell extracts following co-culture of *A. castellanii* with MA104 cells infected with RV Wa by RT-PCR. 2) *A. castellanii* (ATCC50370) fed on MA104 cells infected with RV Wa; 3) positive control RV Wa stock [1062 bp]; 4) *A. castellanii* alone 5) primers alone negative control. M) 100 bp DNA ladder.

3.2.3 Confirmation of virus identity

The RT-PCR products of the viral stocks used in the co-culture of virus and *Acanthamoeba* experiments were sequenced in order to confirm the identities of the viruses used in the study. The amplified and sequenced DNA was checked on the GeneBank website at http://blast.ncbi.nlm.nih.gov/blast.cgi which confirmed the identity of the strains used (see appendix 1).

Primers were obtained to amplify the VPg and protease gene regions of the Coxsackievirus B3 Nancy strain by RT-PCR (Figure 3.39) and the sequence obtained (see appendix 1) from the isolated viral cultures was confirmed to be 100% identical to the published sequence of this virus (Klump *et al.*, 1990).

Figure 3.39. Schematic drawing showing the position of the sequenced RT-PCR product in the genome of Coxsackievirus B3 Nancy strain .

The product obtained from the amplification of the nucleic acid sequence of the viral preparation of CVB5F used in the co-culture of CBV5F and *A. castellanii* was amplified and sequenced (see appendix 1) and was confirmed to be identical to the published sequence of CVB5F using the gene bank facility. The position of the amplified gene includes part of the polymerase genome of the virus, as can be seen in Figure 3.40 (Klump *et al.*, 1990).

Figure 3.40. Schematic drawing showing the position of the sequenced DNA RT-PCR product in the viral genome of coxsackievirus B5 Faulkner strain .

The nucleotide sequence of the RT-PCR product of poliovirus type 2 (see appendix 1) also gave a 100% match when blasted against the published sequence - the position of the amplified region extends across the VP4 region of the PV-2 genome, as can be seen in Figure 3.41 (Klump *et al.*, 1990).

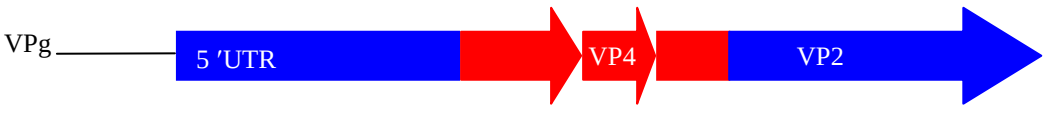


Figure 3.41. Schematic drawing showing the position of the sequenced DNA RT-PCR product in the viral genome of poliovirus type 2 strain .

The RT-PCR product of rotavirus Wa used in the co-culture experiments was purified and sequenced as described in chapter 2 section 2.10. The sequenced nucleotides (see appendix 1) gave a 100% match with the RV Wa genome segment 9 that encodes the outer capsid glycoprotein VP7 (Estes, 2001).

3.3 Solar disinfection of water contaminated with viruses

3.3.1 Inactivation of viruses by SODIS

The effect of solar disinfection of water contaminated with CVB3N was assessed using cell culture to determine the inactivation rate of the virus which is shown in Figure 3.42.

The experiment was performed in triplicate and on 3 different days at 30°C. The titre of viral infectivity after SODIS was compared to controls that were covered by aluminium foil. As shown in Figure 5.1 a reduction by 3 $\log_{10}$ after 1 hour and by 6 $\log_{10}$ after 6 hours was observed, which indicates that viral infectivity decreases with time when samples are subjected to solar irradiance. Moreover, when 250 µM of riboflavin was added to the water contaminated with CVB3N, a 6 $\log_{10}$ reduction of viral infectivity was observed after only one hour showing the marked enhancement of disinfection obtained with riboflavin.

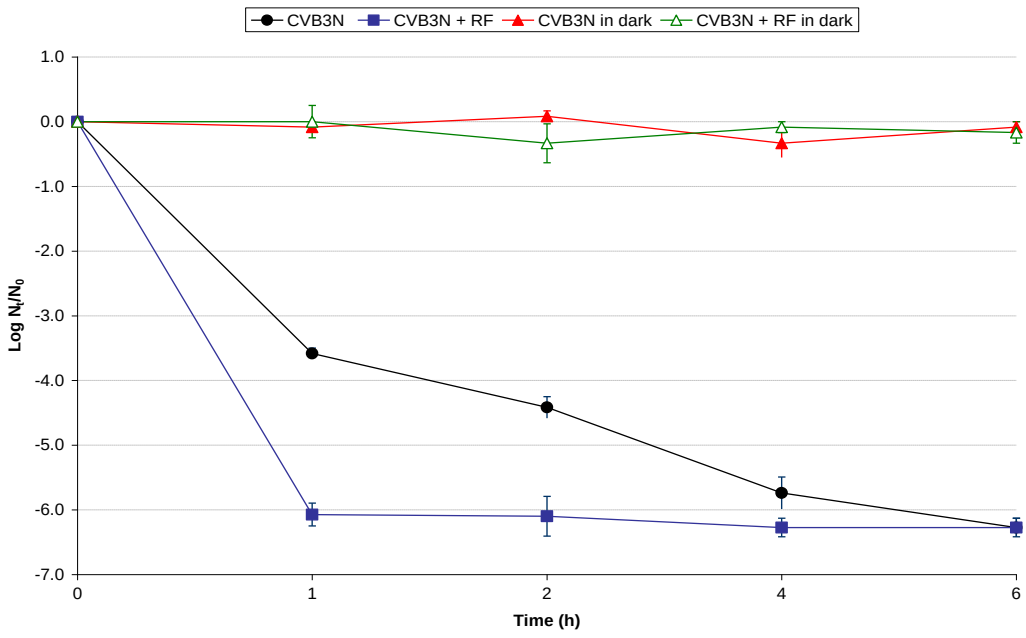


Figure 3.42. The titre of coxsackievirus B3 Nancy strain (CVB3N) exposed to solar irradiance of ~75 W/m2 alone (—●—) and with riboflavin 250 µM (—■—) at 30°C and of controls covered by aluminium foil, n = 9.

In a second experiment (Figure 3.43), after 1 h of solar irradiance, CVB5F infectivity was reduced by 1 $\log_{10}$ and it continued to fall to more than 5 $\log_{10}$ after 6 hrs. This was in contrast to a slight reduction in viral infectivity of about 0.5 $\log_{10}$ in the control

experiments after 6 hrs. However, a faster reduction of viral infectivity was observed when 250 µM of riboflavin was added to the contaminated water - a reduction of almost 6 $\log_{10}$ was observed after 1 h of solar exposure.

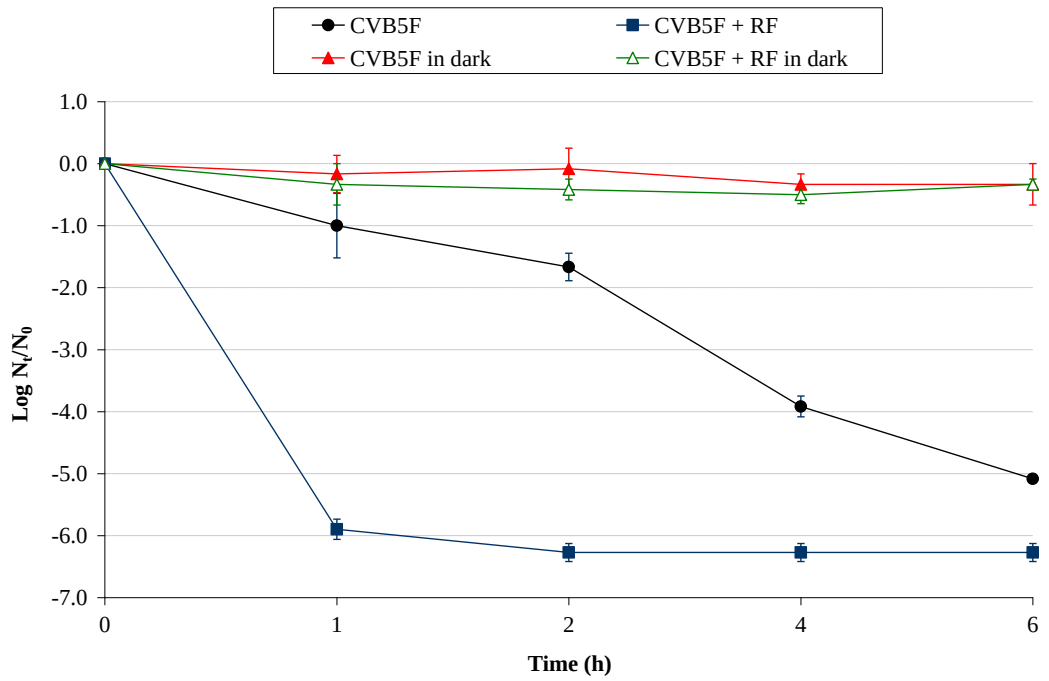


Figure 3.43. The titre of coxsackievirus B5 Faulkner strain (CVB5F) exposed to solar irradiance of ~75 W/m2 alone (—●—) and with 250µM of riboflavin (—■—) at 30°C and of controls covered by aluminium foil, n = 9.

Compared with the results for the two strains of coxsackievirus, PV-2 showed a slower reduction in viral infectivity after solar exposure (Figure 3.44). A reduction of less than 2 $\log_{10}$ was seen after 2 hrs exposure, rising to over 4 $\log_{10}$ after 6 hrs. As with the other viruses, there was a marked improvement following addition of 250 µM riboflavin with PV-2 infectivity being reduced faster by over 2 $\log_{10}$ after 2 hr of solar exposure and 6 $\log_{10}$ after 6 hrs.

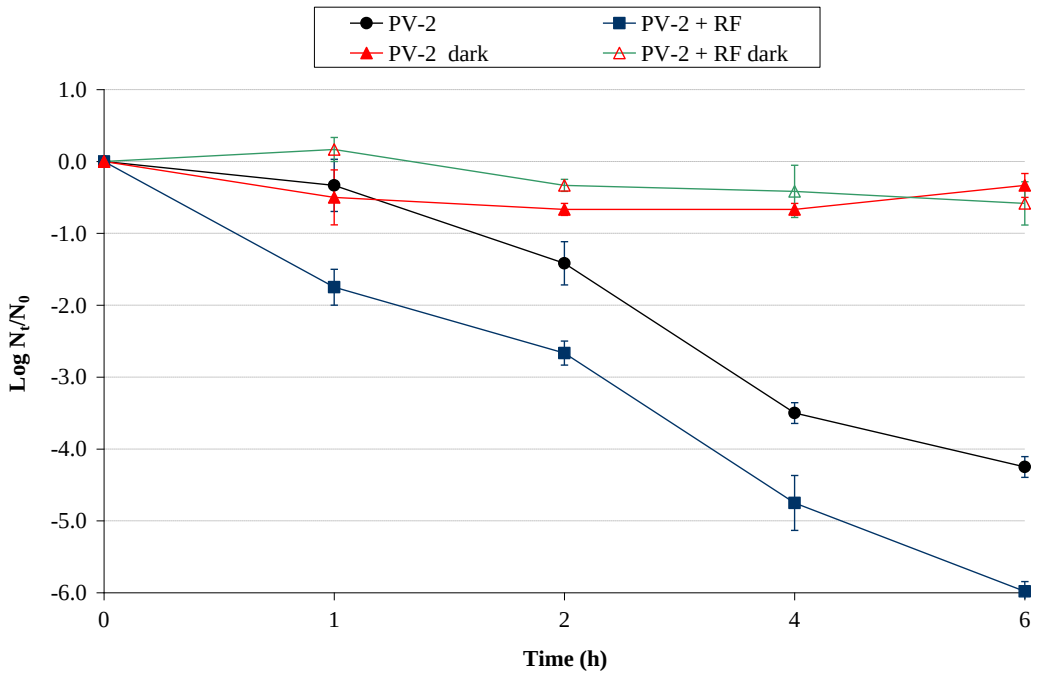


Figure 3.44. The titre of poliovirus type 2 (PV-2) exposed to solar irradiance of ~75 W/m2 at 30°C alone ( ) and with 250 µM of riboflavin ( ) and of controls covered by aluminium foil, n = 9.

3.3.2 Inactivation of viruses by SODIS in the presence of amoeba

When solar disinfection of viruses was performed in the presence of amoeba the results were directly comparable to those of disinfecting viruses on their own. Figure 3.45 shows that the titre of CVB3N co-cultured with *A. castellanii* was reduced by over 3 $\log_{10}$ after 1 hr of solar irradiance and 6 $\log_{10}$ reduction in infectivity after 6 hrs (compare with Figure 3.42).

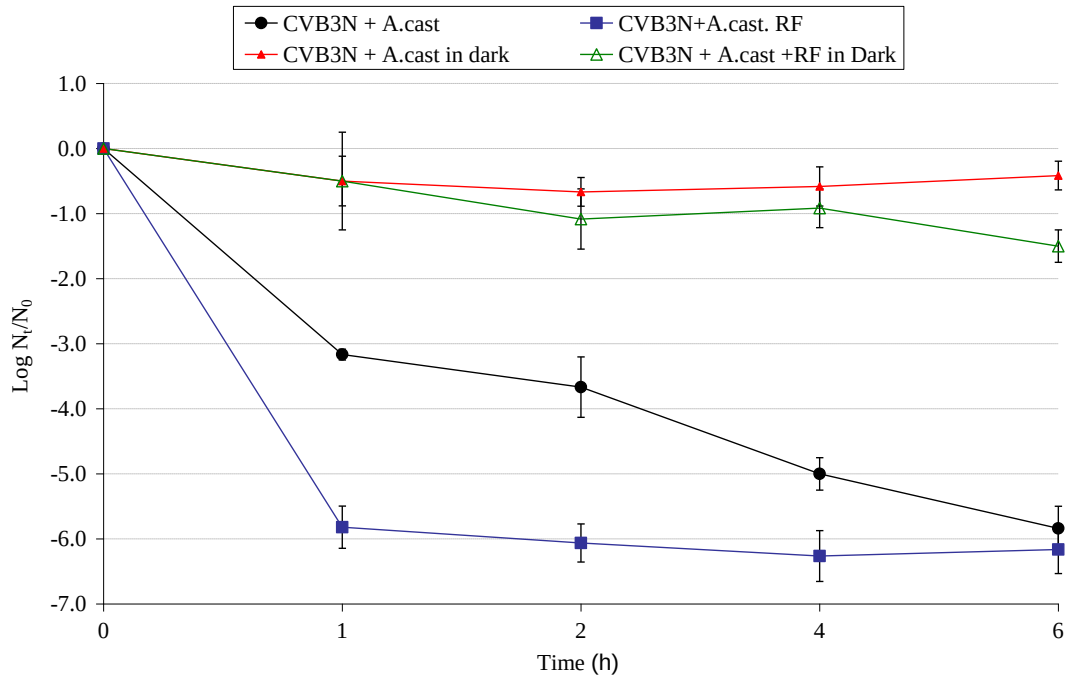


Figure 3.45. The titre of coxsackievirus B3 Nancy (CVB3N) co-cultured with *A. castellanii* and exposed to solar irradiance of ~75 W/m^2 at 30°C with (■) and without (●) 250 µM of riboflavin and of controls covered by aluminium foil, n = 9.

Figure 3.46 shows the infectivity of CVB5F when co-cultured with *A. castellanii* and exposed to solar irradiance. Viral infectivity was reduced by less than 1 $\log_{10}$ after 1 h of solar irradiance and continued to drop until it was reduced by over 5 $\log_{10}$ after 6 h. Again, it was reduced faster with the addition of riboflavin, falling by over 5 $\log_{10}$ in the first hour and by over 6 $\log_{10}$ in the full 6 hours. The results are very comparable to those with virus alone (Figure 3.43).

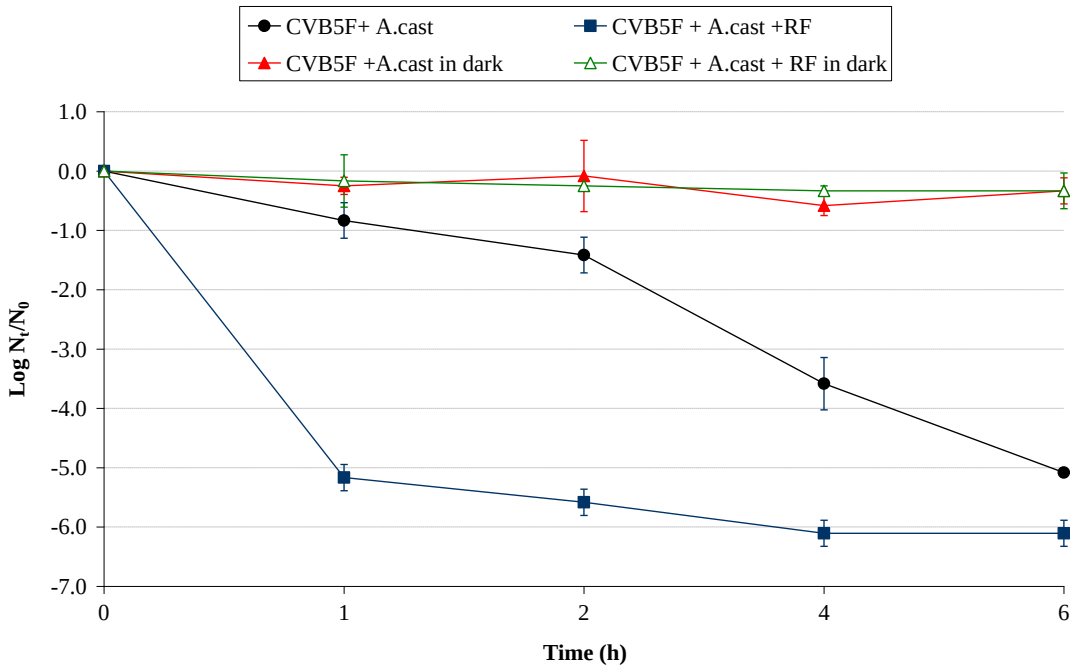


Figure 3.46. The titre of coxsackievirus B5 Faulkner (CVB5F) co-cultured with *A. castellanii* and exposed to solar irradiance of ~75 W/m^2 at 30°C with ( ) and without ( ) 250 µM of riboflavin and of controls covered by aluminium foil, n = 9.

Finally, Figure 3.47 shows the reduction of viral particles of PV-2 when co-cultured with *A. castellanii*. As with PV-2 alone, viability was reduced less rapidly than in the other viruses, by less than 1 $\log_{10}$ after 1 hr of solar irradiance. After 6 hrs the number of viral particles was reduced by less than 4 $\log_{10}$. Once more, reduction in infectivity was faster with the addition of riboflavin, falling by more than 1 $\log_{10}$ after 1 hr and by over 5 $\log_{10}$ after 6 hrs from the start of the experiment.

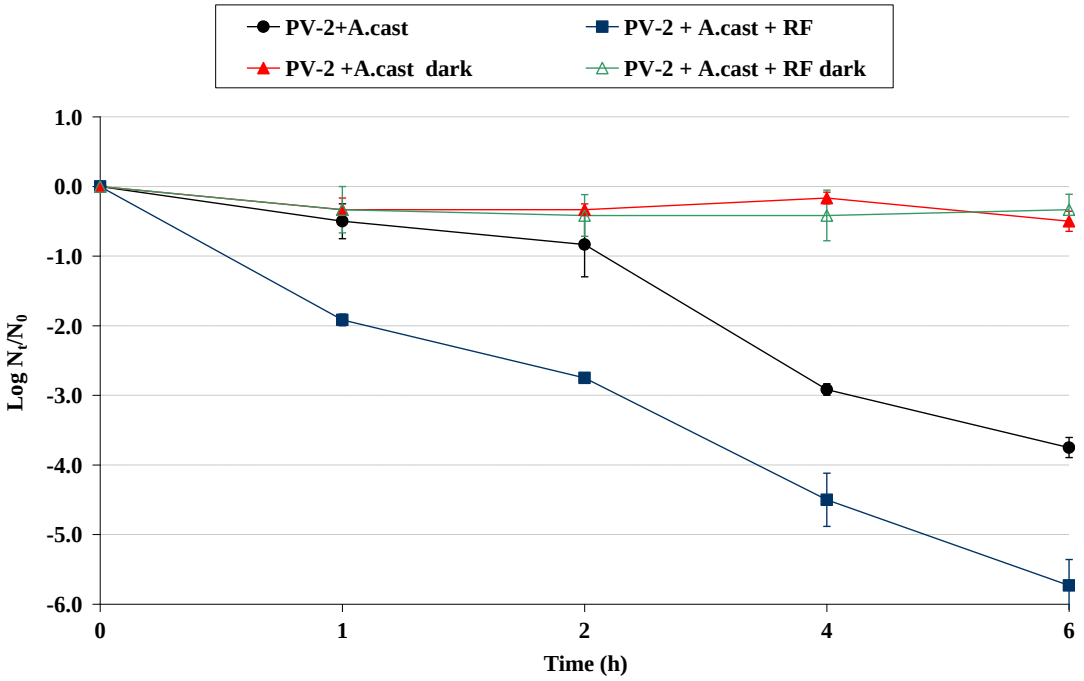


Figure 3.47. The titre of poliovirus type 2 (PV-2) co-cultured with *A. castellanii* and exposed to solar irradiance of ~75 W/m^2 at 30°C with ( ) and without ( ) 250 µM of riboflavin and of controls covered by aluminium foil, n = 9.

Chapter Four: General discussion and conclusions

4.0 Discussion

The first aim of this study was to determine the interaction of free-living protozoa (*A. castellanii*, *A. polyphaga* and *T. pyriformis*) with water-borne human pathogenic viruses (CVB3N, CVB5F, PV-2 and RV Wa). A preliminary method was designed to co-culture PV-2 with *A. polyphaga* and *T. pyriformis* and detect the presence of the virus using electron microscopy (EM). The effect of free-living protozoa on the survival of the viruses following their co-culture, was assessed using a mammalian cell line (Hep-2) to detect the production of CPE. In addition, an indirect immunofluorescence assay (IFA) with specific antibodies was implemented to identify the location of internalised viruses within the *A. castellanii* ATCC 50370. IFA and in vitro cell culture were also used to detect the presence of viruses following co-culture and *A. castellanii* encystment. Reverse-transcriptase polymerase chain reaction (RT-PCR) to detect viral RNA was used to confirm results. The second aim was to determine the impact of solar disinfection (SODIS) on water contaminated with viruses, either alone or when co-cultured with *A. Castellanii*, and in addition, to determine the efficacy of an enhancer on the rate of inactivation of these viruses by the addition of riboflavin prior to SODIS.

The results did not indicate any association of the freely suspended water-borne human pathogenic viruses with the free-living protozoa (*A. castellanii*, *A. polyphaga* and *T. pyriformis*) following their co-culture together. However, CVB3N and RV Wa were detected successfully associated and internalised in *A. castellanii* when virus-infected mammalian cells were engulfed by *A. castellanii*. The SODIS results did not reveal any internalisation or protection of viruses by *A. castellanii*, since a reduction of the viral infectivity comparable to the viruses alone was observed. However, the addition of an enhancer, riboflavin, reduced the viral infectivity to a greater degree and more quickly than when it was absent.

It has been reported that amoeba could provide an important reservoir for water-borne human pathogenic viruses (Mattana *et al.*, 2006). In 1981, Danes and Cerva tried to artificially mimic the wastewater environment that is shared by viruses and free-living protozoa. Their experiment involved mixing both *Acanthamoeba castellanii* with each of five enteroviruses (polio 1, 2, 3; echovirus 4 and 30) using 2% Bacto-Casitone medium in distilled or sewage autoclaved water at pH 6.8. There was a parallel decline of viral infectivity, with or without amoeba. Furthermore, neither internalisation nor attachment was observed by any of the viruses co-cultured with *A. castellanii*, either in

the supernatant or in the sediment of various media at 37°C over 21 days. Amoeba replication was not affected by the presence of any of the viruses tested, and the decline in the number of amoeba was less in the medium containing wastewater. The decline of echovirus 30 infectivity in the medium alone was found to be higher than that for echovirus 30 suspended with amoeba in Bacto-Casitone in wastewater (Danes and Cerva, 1981). Therefore, although no internalisation or attachment of viruses to the amoeba was found, viral survival did appear to be enhanced by co-culture with amoeba. In addition, Baron *et al.* (1980) co-cultured *A. castellanii* at 35°C with either PV type 1 or vesicular stomatitis virus. Following the encystment of the co-cultured *A. castellanii*, cysts were disrupted and inoculated onto African Buffalo Green Monkey kidney (BGM) cells. No plaque forming units were found, and they concluded that *A. castellanii* in this system was not able to act as a carrier for animal viruses (Baron *et al.,* 1980). In 1984, Danes and Cerva performed a similar experiment, co-culturing PV (1 and 2) and echovirus 30 viruses with *Tetrahymena pyriformis*. They again confirmed that the viral infectivity found in the sediment containing the free-living protozoa was maintained longer than viruses in the supernatant, although viral internalisation in free-living protozoa was not assessed (Danes and Cerva, 1984).

In 2006, Mattana *et al.*, tested the role of the interaction of *Acanthamoeba castellanii* with coxsackievirus B3, to determine the role of this free-living protozoon in the survival of the virus. After co-culturing them in a small volume (500 μl) of RPMI 1640, they found that the virus attached after one hour, and was internalised in *A. castellanii* after 24 hours of incubation at 25°C and 37°C, as detected using confocal microscopy. They also reported that the internalised virus persisted inside encysted *A. castellanii* in PBS buffer for six months, and were still infectious following recovery (Mattana *et al.,* 2006). In further experiments, different adenovirus strains have been reported to be associated with *Acanthamoeba* spp. isolated from water sources in the Canary Islands (Lorenzo-Morales *et al*., 2007). The DNA of different adenovirus strains was detected using polymerase chain reaction (PCR), within different types of *Acanthamoeba*. The most common adenovirus found was HAdV-2 associated with *Acanthamoeba* genotype T4, while the adenovirus serotype associated with ocular infection was most frequently associated with *Acanthamoeba* genotype T3 (Lorenzo-Morales *et al*., 2007). Thus, in contrast to earlier work, both of these more recent studies concluded that *Acanthamoeba*

could be considered as a possible reservoir of adenovirus and coxsackievirus with potential transmission to humans and other hosts.

4.1.1 Effect of amoeba on virus titre in #6 basal medium

None of the co-culture methods (using viral titre of 10^7 and 10^5 $TCID_{50}$/ml co-cultured with *Acanthamoeba* 10^4 cells/ml) had affected the viral titre throughout the duration of the experiment - i.e. the viral number did not increase or decrease compared to virus-alone controls, as tested by inoculation of samples on mammalian cell lines. These results indicated that the tested free-living protozoa do not feed on the viruses. This could be due to the ability of *Acanthamoeba* to discriminate between what it can ingest, and what it cannot as described by Bowers and Olszwewski (1983). The feeding of free-living protozoa on, or their ability to internalise other microorganisms is dependent on a number of factors, such as size, movement and physical status (de Moraes and Alfieri, 2008). This can be seen in the relationship of *Acanthamoeba* with either *Pseudomonas fluorescens* or *Proteus mirabilis*. During the movement of *P. fluorescens* using its flagella, when it touches the *Acanthamoeba* it attaches to it and the protozoan engulfs it (Preston and King, 1984). Second, the size of the other microorganism (prey) in relation to *Acanthamoeba* is important, where the protozoa selection of prey is dependent on size, with a reported preference of prey size between 0.8 and 1.2 µm (Chrzanowski and Simek, 1990). In addition, different *Acanthamoeba* species (La Scola *et al*., 2008; Suzan-Monti, *et al*., 2007) are frequently reported to internalise mimivirus; this virus is known to be large in size (700 nm diameter) (Claverie *et al.*, 2009) when compared with other viruses (30-70 nm), e.g. picornaviruses and rotavirus (Claverie *et al.*, 2009; Fong *et al.*, 2005). This size difference could be one of the key factors that enhance the recognition of viruses or microorganisms by *Acanthamoeba*. Another factor that could explain these results is the difference between mimivirus and the other viruses used in this study. The mimivirus genome not only differs by the genome type, which is DNA, but also its genome is large in size (1.2 Mbp) (La Scola *et al*., 2008) when compared with picornaviruses (ssRNA, 7.5 kbp) (Pallansch and Roos, 2001), or even with rotavirus genome (dsRNA 18,556 kbp) (Estes, 2001).

4.1.2 Assessment of PV-2 internalisation in free-living protozoa by EM

PV-2 was not visualised in either of the free-living protozoa, *A. polyphaga* and *T. pyriformis.* These negative results are supported and confirmed by a past study examining poliovirus particles with EM, as reported by Boublik and Drzeniek (1976).

4.1.3 Determination of virus internalisation by amoeba by incubating *A. castellanii* trophozoites with freely-suspended virus

The results from this study, showed that *A. castellanii* (ATCC 50370) did not internalise any of the freely-suspended viruses (CVB3N, CVB5F, PV-2 and RV Wa), after replicating the experimental method of Mattana *et al.,* (2006) by co-culturing both *A. castellanii* and CVB3N. These results are in contrast to the study findings by Mattana *et al.,* (2006), who reported that coxsackievirus was attached to and internalised in *A. castellanii*. The results of this study were confirmed by three types of tests: IFA, mammalian cell line culture and RT-PCR. The findings of the current study are supported by other studies (Danes and Cerva, 1981; 1984; and Baron, 1980), reporting that none of the tested viruses (e.g. PV and echovirus) were internalised in the free-living protozoa utilized (including *A. castellanii* and *T. pyriformis*). The data herein are also in agreement with the study of Mattana *et al.,* (2006) who showed that echovirus-30 was not internalised in *A. castellanii*, even though an identical procedure was applied for the internalisation of coxsackievirus. These negative results are a definitive observation: the internalisation does not occur with the *A. castellanii* strain (ATCC 50370) T4 genotype used in this study. It could also be because the strain used by Mattana *et al.* (2006) was isolated from a patient with an ocular infection, which is a wild strain that could have a number of dissimilarities. The strain used by Mattana *et al.,* (2006) was not deposited at ATCC, so it could not be obtained for confirmation. Yan *et al.,* (2004) found differences between wild type and the laboratory cultured *A. castellanii*; wild type contained more vacuoles and fewer mitochondria than the laboratory type (Yan *et al.,* 2004). The wild type was able to engulf *M. marinum*, *M. smegmatis* and *L. pneumophilia*, and the laboratory types did not engulf *Mycobacterium* and engulfed only *L. pneumophilia*, although both amoeba types internalise latex beads equally.

There are further differences in *Acanthamoeba* strains, as pathogenic or non-pathogenic strains have occurred within the same species (De Jonckheere, 1980). In addition, Wright *et al.* (1981) reported that different strains of *Acanthamoeba* have different rates of feeding on *Cyanobacteria*, and this divergence could be the reason, or one of the reasons, for the difference in results between this study and that reported by Mattana *et al.,* (2006). There are also significant differences between strains within the same species. It was reported by Khan and Tareen (2003) that there are differences in morphology and pathogenicity between the two strains of *Acanthamoeba polyphaga* (ATCC 30871 and CCAP 1501/3c) (Khan and Tareen, 2003). In a morphological analysis, they showed that the cyst of the ATCC strain has six arms, while that of the CCAP 1501/3c strain has five arms, and the ATCC strain showed pathogenicity in corneal epithelial cells, while the other strain did not. Furthermore, *Acanthamoeba* can differentiate between what it can and cannot digest, as reported by Bowers and Olszewski (1983). Since the organism has the ability to differentiate between digestible and indigestible particles, it may also discriminate between engulfed particles based on size or chemical composition. This trend can be seen in the numerous reports on *A. polyphaga* showing that it internalised mimivirus, but not PV-2 or echovirus (Baron *et al.,* 1980; Danes and Cerva, 1984; Mattana *et al*., 2006; Suzan-Monti, *et al*., 2007). It is important to mention here that mimivirus, which causes pneumonia and was isolated from lungs (Khan *et al.,* 2007; Raoult *et al.,* 2007), was internalised frequently in *Acanthamoeba* species (e.g. *A. polyphaga* and *A. Castellanii*) (La Scola *et al*., 2008; Suzan-Monti *et al*., 2007), while only a single study reported that CVB3N was internalised in *A. castellanii* (Mattana *et al.,* 2006). This is in agreement with other authors, e.g. Thomas *et al.* (2010), who reported, "To our knowledge, there is no other report of a well-known pathogenic virus being able to survive inside FLA." (Thomas *et al.,* 2010).

Danes and Cerva (1984) pointed out the numerous difficulties of co-culturing viruses with free-living protozoa, and they indicated that it is not possible to determine the most appropriate titre of virus or the number of amoeba cells in the co-culture. They also highlighted another problem, determining the most suitable method of virus-amoeba co-culture, besides the innate difficulty in imitating the natural environmental conditions (Danes and Cerva, 1984). It is possible, therefore, that this study did not encourage virus uptake due to the presence of endosymbiotic bacteria with *A. castellanii* (ATCC 50370). A new physiological issue that was only discovered in 2011

was the presence of the *Mycobacterium* within the *A. castellanii* (ATCC 50370), which was identified by chance (Glaser *et al.,* 2011). The presence of this bacterium within *A. castellanii* might have a role in the differences between this study and the findings of Mattana *et al.,* (2006). Potentially, *A. castellanii* may only be occupied by one microorganism at a time (Iovieno *et al.,* 2010). The *mycobacteria* could have been treated with antibiotic prior to the start of the experiment, if this had been known at the beginning of the study.

It is known that protozoa have a preference of prey size typically between 0.8-1.2 µm for bacteria (Chrzanowski and Simek, 1990), and consequently the finding that freely-suspended virus particles are not internalised may not be so surprising. It is possible that the observations of Mattana *et al.* (2006) were in fact due to the internalisation of coxsackievirus via the mammalian cells they used to propagate the virus (this mechanism has been demonstrated in the current study). The details of their experimental procedure as reported do in fact leave this possibility open.

4.1.4 Indirect immunofluorescence detection of viruses

Following direct viral infection of mammalian cells, viral particles were successfully detected using IFA. However, following the co-culture of *A. castellanii* with each of the viruses (CVB3N, CVB5F, PV-2 and RV Wa) using the same method as Mattana *et al.,* (2006), no viral particles were visualised by IFA, indicating that no virus was attached or internalised in *A. castellanii*. Although Mattana *et al.,* (2006) used a confocal immunofluorescence microscope, in this study IFA was used, because the main aim was to detect the presence of viral particles. This methodology is supported by Akya *et al.* (2010) who reported that intracellular bacteria could be visualised by a fluorescence microscope, following indirect immunofluorescence staining using an antibody labelled with FITC (Akya *et al.,* 2010). In addition, viruses could also be visualised fluorescing under a fluorescence microscope when using specific antibodies labelled with FITC (Leland and Ginocchio, 2007).

4.1.5 Detection of viruses in *A. castellanii* fed on infected mammalian cells.

Both CVB3N and RV Wa were detected within *A. castellanii* that fed on mammalian cells infected with these viruses using IFA, and showed CPE on mammalian cells. These results indicated that the viruses detected were associated and most probably were internalised within *A. castellanii* and still infective. As reported previously that *Acanthamoebae* were documented by this study to ingest and feed on mammalian cells including Hep-2 cells and reported by other studies (Cursons and Brown, 1978; Rendon-Maldonado *et al.,* 2003).

These interesting results in this study are reported for the first time, showing the presence of CVB3N and RV Wa within *A. castellanii* when it fed on mammalian cells infected with these viruses. This type of internalisation is a unique observation; no one has before seen or published these experiments. The results were proven by three types of tests (IFA, RT-PCR and mammalian cell culture), which gives further confirmation of these results and shows it is not just a coincidence. It would be a potential hazard in the environment when viruses in infected cells are released from an infected person. The ingestion of virus-infected mammalian cells by *Acanthamoeba* is a newly discovered potential hazard. This is similar to the case of people infected with rotavirus, where one of the symptoms is diarrhoea that returns rotavirus captured in the infected epithelial cells back to wastewater (Dotan and Mayer, 2003). These infected cells could be ingested by *Acanthamoeba* or other free-living protozoa present in the wastewater, and then transmitted and protected within free-living protozoa. These results provide a new interaction outcome for the possible interaction between microorganisms in the environment, or in a shared host. These results are in agreement with Pindak *et al.* (1989) who reported that mammalian cells were infected with reoviruses and herpes simplex virus which were fed by *Trichomonas vaginalis*. They also indicated that *T. vaginalis* only digested the mammalian cells. Following the digestion of cells by *T. vaginalis*, the viruses were retained in vacuoles. In addition, the viruses were still infectious nine and six days, respectively, following their recovery from *T. vaginalis*. This indicates that viruses are not a prey type for the protozoan, which did not feed on the viruses although it was in the trophozoites. This internalisation was prolonged at the beginning of the detection by the adjustment of the co-culture pH to neutral, and the

addition of a new inoculum of protozoa. More recently, a more severe virus was reported to be internalised by *T. vaginalis* when it fed on virus infected-lymphocytic cells. The human immunodeficiency virus -1 (HIV-1) was detected in *T. vaginali*s by indirect IFA (Rendon-Maldonado *et al.,* 2003); the authors were able to visualise the FITC labelled antibody attached to the HIV-1 using an immunofluorescence microscope. The timing of detection of viruses showed differences in the results. The optimum time showed stronger fluorescence was up to eight hours following the addition of protozoan cells to the virus-infected cells. After 24 hours, the fluorescence was much weaker than at eight hours. This could explain the negative results observed when mammalian cells infected with both CVB5F and PV-2 were co-cultured with *A. castellanii*, and also the non-repetitive results of CVB3N and RV Wa. Although previous studies used *T. vaginalis* and this study utilised *A. castellanii*, both protozoa share the capability of feeding on mammalian cells. Other cases have reported that infected cells are discharged outside the body of the infected individual; these cells are the ciliated cells in the nasal epithelium layer, as caused by infection with rhinovirus, leading to cold symptoms (Turner *et al.,* 1982). Infection was not severe during the examination, which means that this virus was shedding silently, where the rhinovirus could end up in sewage water post-washing, presenting a ready meal for free-living protozoa in the surrounding environment that are looking for nutrients. Similarly, in hamsters infected with *Mycoplasma pneumonia*, bacteria occurred in the sloughed epithelial cells in the hamster sputum (Muse *et al.*, 1976). This type of case increases the danger of spreading the disease with other animals or humans consuming water from a shared source. Muse *et al.* (1976) also pointed out that similar cases were found in people infected with influenza pneumonia and *M. pneumoniae*.

This study and the later studies raise the possibility for protozoa to internalise viruses, including deadly viruses indirectly in the environment, which could reveal a new mode of viral protection from disinfection. Virus within infected mammalian cells sloughed into wastewater could be protected from disinfection treatment systems that are aimed directly at microorganisms. For example, disinfection by ozone that can completely kill freely suspended viruses at 0.08 mg/l (Emerson *et al.,* 1982), does not kill viruses in infected mammalian cells, requiring more than 4 mg/l of ozone to be inactivated.

The results of the current study have shown that viruses could be internalised in free-living protozoa via ingestion of virus-infected mammalian cells. Pindak *et al.* (1989) reported that mammalian cells infected with reoviruses and herpes simplex virus could

be internalised by *Trichomonas vaginalis*, a non-environmentally transmitted parasite; they also indicated that *T. vaginalis* did not internalise free virus particles. Similar findings have been reported for the human immunodeficiency virus -1 (HIV-1) and *T. vaginalis* (Rendon-Maldonado *et al.*, 2003). This could potentially facilitate the person-to-person spread of the implicated viruses. *Acanthamoeba* possesses an environmental mode of transmission.

Internalisation of viruses in protozoa via infected cells could occur in the environment when these cells are released from an infected person. For example, in infections with rotavirus, the virus can be found in sloughed-off epithelial cells in diarrhoea (Dotan and Mayer, 2003), which might eventually be transported to wastewater. Turner et al. (1982) observed rhinoviruses in infected cells sloughed and discharged from infected individuals; these could potentially enter the drainage system when the infected person washes. These infected cells could be ingested by *Acanthamoeba* or other free-living protozoa present in the water system or wastewater, and then disseminated through the aquatic environment. It has been reported that when phage-infected aquatic bacteria are ingested by protozoa, some of the phage can escape ingestion and be released back into the environment (Clarke, 1998). Viruses ingested by protozoa could possibly also be taken by the protozoa into biofilms and survive within this protected niche environment. When viruses escape from the biofilm, they present a potential hazard and can infect a new host, thus perpetuating the disease cycle (Skraber *et al.*, 2005). It could also be postulated that viruses might replicate within the infected mammalian cells before the cells are degraded by the protozoa, or that multiplication of the *Acanthamoeba* itself may lead to division of infected cell clumps between the progeny, thus increasing the number of carriers, and thus the potential for virus spread.

In addition, *Acanthamoeba* could provide protection from chemical disinfection in water sanitation systems, as demonstrated for bacteria (Kilvington and Price, 1990; Thomas *et al.*, 2008). Emerson *et al.* (1982) showed that cell-internalised viruses require a higher dose of ozone for inactivation than free virus, where sloughed virus-infected mammalian cells could enhance that protection. The findings of the current study open up a range of scenarios that could be studied to further elucidate the implications of this new potential mechanism for virus dissemination.

4.1.6 Detection of infective viral particles by cell culture

To confirm the results of the IFA experiments and to determine the presence of infectious viruses, aliquots of the *A. castellanii*/virus co-cultures were inoculated onto mammalian cells following freezing/thawing. The results showed that none of the freely suspended viruses co-cultured with *A. castellanii* produced any CPE following incubation at 37°C. These results indicated the absence of infective viruses in the co-culture suspension. These data are supported by the study on echovirus-30 by Mattana *et al.,* (2006), which was not internalised using a parallel experiment for the infection of *A. castellanii* with coxsackievirus in their laboratory. On the contrary, both CVB3N and RV Wa produced CPE in the mammalian cell line, indicating the presence of infective viral particles. This is supported by the production of CPE by reovirus and HSV that were inoculated on mammalian cells and fed by *T. vaginalis* (Pindak *et al.*, 1989).

4.1.7 Assessment of viral internalisation following encystment of *A. castellanii*

A number of *A. castellanii* encystment methods (Neff's encystment medium, $MgCl_2$, Taurine, PBS), varying in time and procedure, were used in this study. The comparison between the encystment media indicated that Neff's encystment medium, PBS and addition of $MgCl_2$ to the PYG medium were the most suitable media with regard to procedure and encystment time (five to seven days). Whereas, amoeba encystment method using Taurine agar was laborious and inefficient with regard to the use of consumables. The encystment of *A. castellanii* with freely suspended viruses was performed to determine whether encystment may play a role in allowing viral survival and internalisation in amoeba as reported by Mattana *et al.,* (2006), where in contrast to the results described by this group, no viruses were obtained.

The outcome of this study results indicated that the *A. castellanii* (ATCC50370) strain used does not internalise the freely suspended water-borne pathogenic virus types (CVB3N, CVB5F, PV-2 and RV Wa) investigated using the methods described in this study. These data are in agreement with the findings of most other studies that *A. castellanii* does not internalise or attached to these freely suspended water-borne

pathogenic viruses, even under variable media and methods (Baron *et al.*, 1980; Danes and Cerva, 1981; 1984; Mattana *et al.*, 2006). In contrast, water-borne pathogenic viruses could be internalised when *A. castellanii* fed on mammalian cells infected with CVB3N or RV Wa.

4.1.8 Detection of viral RNA by RT-PCR

The use of RT-PCR to detect the presence of viral RNA/DNA has been exploited for many years, and it has been reported that viral titres equivalent to 1 p.f.u. can be detected (Olive *et al.,* 1990). In addition, the method permits specific detection of individual viral strains, as the primers used can be made specific for regions in the viral genome that diverge quite widely (Gouvea *et al.,* 1990; Richter *et al.,* 2006). A RT-PCR method, which gives 100% positivity of positive samples (Saint-Jean *et al.,* 2001) was used to confirm the results of previous experiments (tissue culture and IFA), and it has been documented that it is a valuable and sensitive method for the detection of viral RNA (McDonagh, 2003). Although the immunofluorescence microscope used in the study was not a confocal microscope, the results were confirmed by detection with RT-PCR. It was important to optimise the designed experiments to determine the minimum concentration that can be detected using RT-PCR. This was 10^2 $TCID_{50}$/ml for CVB3N and RV Wa (equivalent to 14 viral units) while the minimum concentration that could be detected for CVB5F and PV-2 was 10^3 $TCID_{50}$/mL. This is in the same range as previous reports using this same method for detecting coxsackievirus A 19 RNA (Nix *et al.,* 2006; Das *et al.,* 2006). It was not possible to detect the presence of freely suspended viral particles following the co-culture experiments with *Acanthamoeba*.

In the experiment shown in Chapter 3, *A. castellanii* was fed on the HEp-2 cell monolayer infected with CVB3N, and with MA104 RV Wa-infected cells, which did show viral particles associated with *A. castellanii*. Engulfed cells were further studied using the RT-PCR method (Figures 3.31 and 3.38). These results verified the presence of viral RNA in the co-culture sample; confirming the viruses that were seen by IFA where an internalisation of CVB3N and RV Wa by *A. castellanii* physically occurred. *Acanthamoeba* co-cultured with mammalian cells and infected with viruses (CVB3N, CVB5F, PV-2 and RV Wa) showed that both CVB3N and RV Wa were internalised and detected by cell culture, IFA, and were subsequently confirmed by RT-PCR.

Although CVB3N and RV Wa were still present within the amoeba fed on the virus-infected mammalian cells. Conversely, following co-cultured amoeba encystment, RT-PCR did not show any product for either CVB3N or RV Wa. Although no viral RNA was detected, it does not rule out the possibility that *Acanthamoeba* could be a carrier for viruses when it engulfs virally-infected mammalian cells in its trophozoite stage. This is reminiscent of what occurs with *Vibrio cholerae* when it is co-cultured with *Acanthamoeba*, where this bacterium was internalised in *Acanthamoeba* when in the trophozoite stage, but following encystment and excystment, the *Vibrio cholerae* were not recovered (Thom *et al.*, 1992).

4.2 Solar disinfection

The second aim, the evaluation of SODIS to inactivate viruses, was carried out to continue the first approach of the internalisation and protection of water-borne human pathogenic viruses in free-living protozoa following co-culture and SODIS. The inactivation of viruses co-cultured with *A. castellanii* using SODIS was investigated and compared with inactivation of viruses only. In addition, the same *A. castellanii* and viruses (CVB3N, CVB5F and PV-2) were used in SODIS experiments, either alone or with the addition of an enhancer such as riboflavin. This was performed because previous studies reported that viral persistence was prolonged in the sediment of dead free-living protozoan cells following co-culture (Danes and Cerva, 1981). It has been also reported that the plasma membrane of *E. coli* was disrupted and became more permeable due to exposure to solar irradiance (Bosshard *et al.*, 2010a).

In the solar disinfection experiments, four experiments were performed for each of three viruses tested. First, viruses were exposed to solar irradiance alone and the viral infection titre was measured using the tissue culture. Second, 250 µM of riboflavin (vitamin B2, with absorbance between 265-370 nm) was added to the viral suspension to determine its effect on the rate and extent of viral inactivation (Ruane *et al.*, 2004). Third, viruses were co-cultured with *A. castellanii* to determine if this would inhibit the effect of SODIS. Fourth, 250 µM riboflavin was added to the *Acanthamoeba*/viral co-culture prior to SODIS. The results of this study demonstrated that solar disinfection of virus-contaminated water was efficient. The co-culture of the amoeba with each of the viruses did not provide any protection to the viruses, since the results were comparable

with data obtained from viruses alone. The novel results are the significant reduction of viral infectivity by the addition of riboflavin.

4.2.1 Solar disinfection of water contaminated with viruses

SODIS results indicated a significant reduction of viral infectivity including coxsackievirus B3N (t test $P \leq 0.012$) and B5F (t test $P \leq 0.012$), and a high reduction of PV-2 (t test $P \geq 0.2$). The results indicated that the reduction of the viral infectivity was due to only SODIS, because the temperature of the water bath did not exceed 34°C. This was a critical step to exclude the effect of heat during the experiment. These findings are supported by other studies on different microorganisms, e.g. faecal coliforms, *E. coli*, lambda phage and bovine enterovirus that were all inactivated under direct sunlight (Reed *et al.,* 2000), dimmed light from short distance (Kumar *et al.,* 2004; Ubomba-Jaswa *et al.,* 2009), or a 150W lamp (McGuigan *et al.,* 1998).

The reduction of infectivity of PV-2 was less than that observed for the other viruses tested in this study. Even at the end of the experiment, after six hours of exposure to solar irradiance at a total optical dose of 1.62 kJ, it was reduced by 1 $\log_{10}$ less than CVB5F, and 2 $\log_{10}$ less than CVB3N. This could be due to differences in the outer capsids of the viruses, since the PV-2 receptor is PVR, while the coxsackievirus receptor is CAR. Differences in the receptor recognition site on the viral capsid could lead to a difference in their absorbance rate for light. In addition, it has been reported that VPg (viral protein genome-linked) is not required for PV-2 infectivity (Nomoto *et al*., 1977).

The solar irradiance inactivates pathogens because UV-A interacts with nucleic acids, proteins and enzymes (Babior, 1997). The reduction of viral infectivity under solar irradiance is due to the reaction of UV-A with dissolved oxygen in the water. This reaction starts with the reduced oxygen accepting one electron, leading to the production of reactive oxygen species. When electrons interact with O_2, they yield superoxide atoms (O_2^-) producing a second reduced product that, once it gains a second electron, results in the formation of hydrogen peroxide (H_2O_2). Following the previous reductions, when a third electron plus H_2O_2 and hydrogen react, hydroxyl radicals (•OH) are produced, and once O_2^- reacts with •OH it produces a singlet oxygen ($^1O_2$) and hydroxide, which are all toxic molecules (Swiss Federal institute of environmental

science and technology (EAWAG)), 2002; Babior, 1997; Howes, 2005; Kohn and Nelson, 2007). The hydrogen peroxide and singlet oxygen are responsible for genome strand separation. Singlet oxygen also reacts with the side chains of the amino acids of the protein and selectively damages particular residues. Furthermore superoxide and singlet oxygen cause lipid peroxidation, leading to its damage, and the oxidation of cholesterol occurs at 4- and 6-carbons (Babior, 1997; Davies, 2003; Imlay and Linn, 1988; Tyrrell and Keyset, 1990). In addition, the actions responsible for inactivation of microorganisms using SODIS were recently documented by Bosshard *et al.* (2010a). The microorganism inactivation mechanism caused by the effect of SODIS on cells was reported to show an aggregation of 80% of the intracellular proteins of *E. coli*, following solar irradiance up to 2500 kJ/m^2. This aggregation of proteins subsequently halted cell functions regarded as vital for cell viability. Other activities are also affected, e.g. transcription and translation, transport systems, amino acid production and degradation, ATP synthesis and glycolysis, which eventually results in the destruction of the microorganisms (Bosshard *et al.,* 2010b). They also indicated that the plasma membrane became more permeable than the bacterial controls. In addition, respiration appeared to be one of the key factors leading to cell inactivation following exposure to UV-A.

In summary, SODIS of contaminated water is a very useful method for disinfecting water for use by humans, animals, and plants that all share the same environment, thus minimising the risk of contamination and the transfer of the pathogen from one host to another (Heaselgrave *et* al., 2006; McGuigan *et al*., 1998). In addition, SODIS would be a beneficial disinfection method for water obtained from rivers, contaminated with pathogens inside epithelial cells from the sputum of people or animals (Muse *et al.*, 1976; Turner *et al.*, 1982).

4.2.2 Solar disinfection of water contaminated with viruses, with riboflavin as an enhancer

There have been few studies reporting the assessment of solar disinfection in association with riboflavin to inactivate viruses (Callahan *et al.*, 2008; Wallis *et al.*, 1969). Treatment of viruses using light with a medium containing riboflavin and calf serum has been investigated on herpes virus, adenovirus, vacciniavirus and poliovirus. These reports indicated that poliovirus was completely photo-resistant (Wallis *et al.*,

1969). Callahan *et al.* (2008) investigated the inactivation of adenovirus and recombinant viruses using long-wavelength ultraviolet irradiation (LWUVI) and 50 µM riboflavin. They reported that adenovirus was inactivated within 20 min. Effective inactivation of human immunodeficiency virus, porcine parvovirus and West Nile virus in platelets in the presence of 50 µM riboflavin, performed using UV light, was reported by Ruane *et al.* (2004). None of the previous studies had investigated the inactivation of non-enveloped RNA viruses like CVB3N, CVB5F and PV-2 in water in the presence of riboflavin, visible light, and UV-A.

The implementation of SODIS with the addition of riboflavin showed a significant reduction of CVB3N infectivity (t test $P \leq 0.013$), CVB5F (t test $P \leq 0.012$), and PV-2 showed great reduction of the viral infectivity. This addition shows that riboflavin has a remarkable effect in the inactivation process (Kumar *et al.,* 2004). Likewise, when riboflavin was added the infectivity of CVB3N was reduced slightly more than that of CVB5F; with a total optical dose of 1.62 kJ, the difference in viral infectivity reduction was comparable to the results without riboflavin. The reduction of viral infectivity is primarily an effect of UV-A on the structure of the protein-based virus capsid, which is degraded by singlet oxygen (Babior, 1997).

Recently, a higher concentration of riboflavin was used, where 250 µM riboflavin inactivated the vegetative and trophozoites forms of bacteria and amoebae and their dormant stage forms including cysts and spores, respectively (Heaselgrave *et al.,* 2010). Only the vegetative forms of microorganisms were totally killed, while the dormant stage was reduced by 3.5 $\log_{10}$. In addition, UV-A excites riboflavin which interacts with DNA and RNA, enhancing the production of singlet oxygen ($^1O2$) and transferring an electron to oxygen to form a superoxide radical ($^{-}O2$). This interaction damages the guanine base, which is one of the four bases that are integral to viral DNA and RNA, and can lead to the breakage of genome strands. The interaction also results in damage to the viral protein capsid, which consequently prevents it from infecting and replicating in host cells (Ito *et al.,* 1993). There is an enhanced effect of the action of UV-A light when it is absorbed by riboflavin, since it interacts with oxygen and produce ORSs (e.g. $^1O_2$ and H_2O_2). These ORSs are the causative agents for nucleic acid damage, especially affecting guanine bases and the protein components of the capsid of virions (Maisch *et al.,* 2007; Kumar *et al.,* 2004; Reddy *et al.,* 2008). As a result, the presence of riboflavin produces more ORSs in the presence of oxygen in the suspension of contaminated water, causing further and faster reduction of viral

infectivity. This process may be enhanced by the fact that one molecule of H_2O_2 is induced when every two molecules of riboflavin are broken by photons (Sato *et al.*, 1995), indicating that a higher concentration of riboflavin can cause a greater viral inactivation rate.

4.2.3 Solar disinfection of water contaminated with viruses co-cultured with *A. castellanii*

The efficacy of SODIS on the ability of *A. castellanii* to internalise and protect water-borne pathogenic viruses from damage was examined following 24 h of co-culture. The results did not show any significant difference (t test $P \geq 0.18$) in the loss of viral infectivity, either when CVB3N was subjected to solar irradiance alone, or when it was co-cultured with *A. castellanii*. Although there was a marginal reduction of viral infectivity of CVB3N when co-cultured with *A. castellanii*, it was not significant. Viral infectivity of CVB3N co-cultured with *A. castellanii* was reduced slightly less than the virus alone without riboflavin, reaching almost a 6 $\log_{10}$ reduction at the end of the experiment, while without *A. castellanii*, it was reduced by over 3 $\log_{10}$ after one hour, and by 6 $\log_{10}$ after six hours.

SODIS is capable of inactivating different viruses by use of a phosphor lamp that yields 265-370 nm, including human immunodeficiency virus, porcine parovirus and West Nile virus (Ruane *et al.* 2004). In the case of CVB5F, the reduction of viral infectivity was almost unchanged either by co-culture with *A. castellanii* or for the virus alone. There was no significant difference in the inactivation rate of CVB5F, as can be seen with the P value in the student (t test $P \geq 0.14$). Viral infectivity of PV-2 co-cultured with *A. castellanii* and exposed to solar irradiance was reduced slightly less than when the virus alone was exposed to solar irradiance, with a difference of 0.5 $\log_{10}$ which was not significant (t test $P \geq 0.09$).

The titres of viruses when co-cultured with amoeba and exposed to solar irradiance for up to six hours showed similar results to viruses alone under the identical conditions, which supports the suggestion that free suspended viruses were not internalised in the *Acanthamoeba*. The trophozoites form of *Acanthamoeba* was reported previously to be totally killed when exposed to SODIS, indicating that it does not have the ability to

protect the viruses from such treatment (Heaselgrave *et al.,* 2010), or to prolong virus survival and infectivity (Danes and Cerva, 1984).

4.2.4 Solar disinfection of water contaminated with viruses co-cultured with *A. castellanii* and riboflavin

These results of the currenbt study indicate that the *Acanthamoeba* trophozoites are sensitive to SODIS (Heaselgrave *et al.,* 2006) and they do not provide the virus with protection from solar irradiance, even if theoretically they were internalised by the effect of light because of the increased permeability due to SODIS (Bosshard *et al.,* 2010). Furthermore, SODIS in association with riboflavin did show a marked reduction in viral infectivity, as has previously been shown with no recovery as reported by others (Heaselgrave *et al.,* 2010; Kumar *et al.,* 2004). Moreover, the results of solar irradiance of PV-2, either alone or when co-cultured with *A. castellanii* mixed with riboflavin, were not significant (t test $P \geq 0.177$).The data of this study could suggest that PV-2 required a higher solar irradiance to be totally inactivated, possibly similar to which was reported previously for PV-2 virus that it was totally inactivated using 850W/m^2 of solar irradiance (Heaselgrave *et al.*, 2006).

In summary, the addition of riboflavin to contaminated water under solar irradiance significantly improved the virus disinfection rate. Riboflavin may be beneficial in cloudy or dusty places where reduced natural sunlight would make UV-irradiation alone impractical. Viral inactivation is suggested to be due to ORSs that originate from dissolved oxygen, and these superoxide free radicals are caused by UV-A at 320-400 nm (near UV) (Joshi *et al.,* 1985; Reed, 1997). Disinfection of contaminated water by the combined effect of solar irradiation and the addition of riboflavin could be the basis for the development of practical treatment methods for contaminated water, particularly where the existing infrastructure is inadequate and low technology methods could be utilised to provide safe drinking water. No special skills are needed for the implementation of SODIS. A one-page instruction leaflet could be sent with bottles (with or without enhancers) to help provide safe drinking water. The current study thus

provides a practical example of low and cheap technology method for production of safe drinking water in various circumstances.

4.3 Conclusions

1. Free-living protozoa do not internalise human pathogenic viruses freely suspended in the acquatic environment.
2. Free-living protozoa can internalise human pathogenic viruses via internalisation of infected cells. This new finding has implications for virus transmission in the environment, as internalisation of sloughed cells containing viruses from infected persons could result in extra protection for these viruses and prolong their survival in the environment.
3. Free-living protozoa do not protect freely-suspended viruses from solar disinfection in aquatic environments. It could be possible that viruses internalised in free-living protozoa via infected cells could be protected by a shading effect, although the protozoa themselves are not resistant to solar iradiation.
4. Addition of riboflavin to an acquatic environment will enhance the effect of solar disinfection against virus infectivity. This could be utilised to provide safe cost-effective and simple water treatment, especially in developing countries.

4.4 Recommendations and future work

- Methods to detect viruses in water should include the investigation of mammalian cells that might contain internalised viruses or other pathogens.
- Disinfection systems should include a procedure to inactivate microorganisms such as adenovirus that are internalised within other cells, e.g. mammalian cells and free-protozoa.
- Future studies could be carried out to confirm that solar disinfection, with and without enhancers, is effective in reducing the viability of *Acanthamoeba* in its free living and encysted forms, e.g. by suspending *Acanthamoeba* trophozoites and cysts in water, and exposing suspension to SODIS. Also assess the implementation of SODIS to minimise or inhibit the production of biofilms in expiremental water systems. *Acanthamoeba* infected with mimivirus would be ideal for investigating whether solar disinfection methods can neutralise internalised viruses. Further experiments could also determine if a SODIS enhancer would provide a greater reduction of viral infectivity.
- Since viruses in virus infected-mammalian cells were internalised in *A. castellanii*, further experiment could determine the efficacy of solar disinfection on viral-infected mammalian cells taken by free living protozoa.
- The interaction of the viruses used in this study with other free-living protozoa could be investigated, e.g. the interaction of other *Acanthamoeba* and *Naegleria* with water-borne human pathogenic viruses.
- Since waste-water has been shown previously to include mammalian cells infected with viruses, further studies could investigate the efficacy of SODIS on water-borne pathogenic viruses internalised in mammalian cells.
- Since water is turbid in some places, a study could performed to determine the efficacy of solar disinfection of water contaminated with pathogenic viruses in turbid water. By adjustment of different concentrations of turbidity of contaminated water and exposing it to SODIS and measure the inactivation rate.
- Further study could also determine the efficacy of solar disinfection of contaminated water with pathogenic viruses under natural sunlight, with and without an enhancer (e.g. riboflavin).

- The potential for *Acanthamoeba* to transfer viruses into biofilms could be investigated, e.g. the ability of trophozoites to trap viral-infected mammalian cells in biofilms.

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Appendices

Appendix 1

Sequences of the amplified viruses genes used in the study showing the nucleotides position in the viral genomes.

> Poliovirus type 2

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611   CATAAAGCGAATTGGATTGGCCATCCGGTGAGTGTTGTGTCAGGTATACAACTGTTTGTT
671   GGAACCACTGTGTTAGCTTTACTTCTCATTTAACCAATTAATCAAAAACAATACGAGGAT
731   AAAACAACAATACTACAATGGGCGCCCAAGTTTCATCACAGAAAGTTGGAGCCCACGAAA
791   ATTCAAACAGAGCCTATGGCGGGTCCACCATCAATTACACTACAATCAATTACTATAGGG
851   ACTCTGCAAGCAATGCAGCAAGCAAGCAAGATTTTGCACAAGATCCGTCCAAGTTCACCG
911   AACCCATTAAGGACGTCCTTATTAAGACCGCTCCCATGCTAAACTCCCCAAACATTGAGG
971   CGTGTGGTTATAGTGACAGGGTAATGCAGCTAACTCTGGGCAATTCAACGATCACCACCC
1031  AAGAAGCGGCCAATTCTGTTGTTGCCTACGGTAGATGGCCTGAATACATCAGAGATACCG
1091  AGGCAAATCCTGTAGACCAACCAACCGAGCCCGATGTAGCCGCGTGCAGGTTCTACACAT
1151  TAGATACCGTCACTTGGCGCAAGGAGTCCAGAGGGTGGT
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>Coxsackievirus B3 Nancy

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5280  TTTGCGGGTTTTCAAGGTGCTTATACAGGAGTGCCCAACCAGAAGCCCAGAGTGCCTACC
5340  CTGAGGCAAGCAAAAGTGCAAGGCCCTGCCTTTGAGTTCGCCGTCGCAATGATGAAAAGG
5400  AACTCAAGCACGGTGAAAACTGAATATGGCGAGTTTACCATGCTGGGCATCTATGACAGG
5460  TGGGCCGTTTTGCCACGCCACGCCAAACCTGGGCCAACCATCTTGATGAATGATCAAGAG
5520  GTTGGTGTGCTAGATGCCAAGGAGCTAGTAGACAAGGATGGCACCAACTTAGAACTGACA
5580  CTACTCGAATTGAACCGGAATGAGAAGTTCGGAGACATCGGAGGCTTCGTAGCCAAGGAG
5640  GAAGTGGAGGTTAATGAGGCAGTGCTAGCAATTAACACCAGCAAGTTTCCCAACATGTAC
5700  ATTCCAGTAGGACAGGTCACAGAATACGGCTTCCTAAACCTAGGTGGCACACCCACCAAG
5760  AGAATGCTTATG
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>Coxsackievirus B5 Faulkner

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6071  TTTGAGGAGGCCATATTCTCAAAATACATTGGAAATGTCAACACACACGTAGATGAATAC
6131  ATGCTAGAAGCTGTTGATCATTATGCCGGGCAGTTGGCCACACTGGACATTAGCACCAAA
6191  CCAATGAAATTGGAGGACGCTGTGTACGGCACCGAGGGTCTCGAAGCCCTCGATCTAACT
6251  ACGAGTGCAGGCTACCCTTATGTTGCATTGGGCATCAAGAAGAGAGACATTCTTTCCAAA
6311  AAGACCAAGGATTTAACCAAGTTAAAGGAATGCATGGATAAATATGGCTTGAACTTGCCA
6371  ATGGTAACTTATGTTAAAGACGAGCTCAGGTCTGCAGAGAAGGTAGCAAAAGGGAAATCC
6431  AGATTGATAGAAGCATCCAGCTTGAATGACTCCGTGGCAATGAGACAAACATTCGGCAAC
6491  CTATACAAAACTTTTCATCTAAATCCAGGGATTGTGACTGGCAGTGCTGTTGGGTGTGAC
6551  CCAGACCTCTTTTGGAGTAAAATACCGGTGATGTTAGATGGTCACCTTATAGCCTTTGAT
6611  TACTCTGGATACGATGCTAGCTTGAGCCCCGTCTGGTTTGCCTGCCTAAAACTATTACTT
6671  GAGAAACTTGGATACTCGCACAAGGAGACCAATTATATTGATTACCTGTGCAACTCCCAT
6731  CACCTGTACAGGGACAAACACTATTTTGTGCGGGGTGGCATGCCTTCAGGATGTTCT
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>Rotavirus Wa strain

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53    ATGGTATTGAATATACCACAATTCTAATCTTTTTGATATCAATCATTCTACTCAACTATA
113   TATTAAAATCAGTGACTCGAATAATGGACTACATTATATATAGATTTTTGTTGATTACTG
173   TAGCATTATTTGCTTTGACAAGAGCTCAGAATTATGGACTTAACTTACCAATAACAGGAT
233   CAATGGACGCTGTATATACTAACTCTACTCAAGAAGAAGTGTTTCTAACTTCTACGTTAT
293   GTCTGTATTATCCAACTGAAGCAAGTACTCAAATCAATGATGGTGACTGGAAAGACTCAT
353   TGTCGCAAATGTTTCTTACAAAGGGTTGGCCAACAGGATCTGTTTACTTTAAAGAGTACT
413   CAAATATTGTTGATTTTTCTGTTGACCCACAGCTGTATTGTGACTATAATTTAGTACTTA
473   TGAAATATGACCAAAGTCTTGAATTAGATATGTCAGAGTTAGCTGATTTAATATTGAATG
533   AATGGTTATGTAACCCAATGGATGTAACATTATACTATTATCAACAATCGGGAGAATCAA
593   ATAAGTGGATATCGATGGGATCATCATGTACCGTGAAAGTGTGTCCGCTAAATACACAAA
653   CGTTAGGGATAGGTTGTCAAACAACAAACGTAGACTCATTTGAAATGATTGCTGAGAATG
713   AGAAATTAGCTATAGTGGATGTCGTTGATGGGATAAATCATAAAATAAATTTAACAACTA
773   CGACATGTACTATTCGAAATTGTAAGAAATTAGGTCCAAGAGAAAATGTAGCTGTAATAC
833   AAGTTGGTGGTTCTAATGTGTTAGACATAACAGCAGATCCAACAACTAATCCACAAACTG
893   AGAGAATGATGAGAGTGAATTGGAAAAAGTGGTGGCAAGTATTTTATACTATAGTAGATT
953   ATATTAATCAAATTGTACAGGTAATGTCCAAAAGATCAAGATCATTAAATTCTGCAGCTT
1013  TTTATTATAGAGTATAGATATATCTTAGATTA
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