Persistence of Infectious *Enterovirus B* in Free-Living Amoebae

by

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Abstract

The persistence of human pathogens, such as *Legionella* bacteria, inside environmental free-living amoebae is emerging as a major waterborne health risk, which could affect pathogen disinfection, removal, and dispersion from engineered water systems. Amoebae are natural predators, which graze on water-based biofilms, where they shape microbial populations. Amoebae are also found in hot water tanks, cooling towers, drinking water outlets, moist surfaces, medical instruments, dental waterline units, and are often linked to nosocomial infections. Amoeba-resistant microorganisms can seek shelter within the disinfection-resistant protozoan cysts and can easily disperse with them through water distribution systems.

Despite the co-occurrence of amoebae and human enteric viruses in similar environments, the association between them is currently poorly understood. This is an important knowledge gap of great public health significance, especially for high-risk enteric viruses (*e.g.* coxsackieviruses, which are associated with Type 1 Diabetes, neonatal neurological impairment, and even miscarriage). Of potential concern to the water industry and public health in general, but not well understood, is virus internalization by amoebae. This could reduce the efficacy of current virus management strategies for contaminated waters.

Here I demonstrate that an infectious clinical *Enterovirus B* isolate (coxsackievirus B5) persisted over time in association with two of the most commonly reported waterborne amoebae (*Vermamoeba vermiformis*, isolated from a hospital cooling tower, and *Acanthamoeba polyphaga*, isolated from human corneal scrapings). My findings also demonstrate for the first time that a human enteric virus could be localized in expelled amoebae vesicles. In addition, infectious virions were also associated with mature *A. polyphaga* cysts for at least 20 months.

Preface

1. Contributions

Parts of this work are currently used for manuscript preparation and involved contributions from other people. Dr. Rafik Dey helped with fluorescence microscopy optimization, and with the staining of *V. vermiformis* cysts at day 0 (Figure 3.10a), which I had previously co-cultured with CVB5. Ms. Candis Scott helped with RT-qPCR training and assay validation (Figure 2.2), where standard curve replicates were prepared by myself. Dr. Qiaozhi Li provided me with adapted Excel spreadsheets for the calculation of $TCID_{50}$ and MPN IU/mL from virus infectivity assays. Virus-amoebae co-cultures used for TEM imaging were prepared by myself and submitted to the imaging core at the Faculty of Medicine and Dentistry, University of Alberta, where they were sectioned and carbon-coated by Dr. Woo Jung Cho.

2. Ethics Requirements

The clinical *Enterovirus B* isolate, provided by Dr. Xiao-Li Pang at the Provincial Laboratory for Public Health (ProvLab) in Edmonton, AB, Canada, had been passaged *in vitro* several times by trained ProvLab personnel before it was provided for the research described here, and was confirmed by ProvLab personnel to contain no trace amounts of biological patient material. Details regarding virus isolation and patient information were not disclosed to me and are exclusively known to trained ProvLab personnel. Therefore, as confirmed with the Research Ethics Office at the University of Alberta, no human research ethics approval was required.

To my parents, Dimitar and Diana, whose endless love, patience, and support mean more than words could ever describe.

To my grandparents, Maria and Georgi, who never stopped being proud.

To my grandparents, Angel and Evdokia, who cannot share my joy, but will always remain in my heart.

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I am very grateful to all members of the Ashbolt lab, for contributing to a very friendly, supportive, and unique scientific work environment, especially Dr. Rafik Dey, Lena Dlusskaya, Rachel Yee, Candis Scott, Dr. Nancy Price, Dr. Qiaozhi Li, and Dr. Alexey Atrazhev, for their help and support.

I am also grateful to Francisca Cristi Muñoz and Theodore dos Santos from Dr. Maya Schmulevitz' lab for help with Western blot assays.

I would like to thank Dr. Xiao-Li Pang (ProvLab) for providing me with the MA104 cell line and with an infectious clinical *Enterovirus B* isolate for my studies, as well as for allowing me to use her ABI 7500 equipment for my RT-qPCR assays. I am also very grateful to Min Cao and other members of the Pang lab.

In addition, my sincere thanks include Dr. Judy Gnarpe, for providing me with invaluable teaching and TA experience and guidance.

I am also very thankful to Dr. Matthias Goette, Dr. Edan Foley, Dr. Kevin Kane, as well as Anne Giles, Tabitha Vasquez, Melissa Northmore, Debbie Doudiet, Michelle Zadunayski, and other MMI staff for their help and support.

Finally, I could barely express my gratitude towards my parents, my extended family, and friends from all over the world, who continuously encouraged me and kept me grounded.

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List of Abbreviations

Acanthamoeba polyphaga Mimivirus – APMV

Adenosine Triphosphate – ATP

American Type Culture Collection – ATCC

Ammonium Iron (II) Phosphate Hexahydrate - $Fe(NH_4)_2(SO_4)_2*6H_2O$

Ammonium Persulfate - APS

Amoeba-Resistant Microorganisms – ARM

Base Pairs – bp

Base Ten Logarithm – Log_{10}

Bovine Serum Albumin – BSA

Calcium Chloride - $CaCl_2$

Candidate Contaminant List – CCL

Carbon Dioxide – CO_2

Casein-Glucose-Yeast-Extract Medium – CGYEM

Central Nervous System – CNS

cis-acting Replication Element – *cre*

Complementary DNA – cDNA

Contractile Vacuole – CV

Coxsackie-Adenovirus Receptor – CAR

Coxsackievirus - CV

Cytopathic Effect – CPE

Day Post Infection - dpi

Decay Accelerating Factor – DAF

Degree Celsius – ^oC

Deoxynucleotide – dNTP

Deoxyribonucleic Acid - DNA

Dimethyl Sulfoxide – DMSO

Disodium Hydrogen Phosphate (Sodium Phosphate Dibasic) - Na_2HPO_4

Dithiotreitol - DTT

Double-Stranded Deoxyribonucleic Acid - dsDNA

Eagle's Minimum Essential Medium – EMEM

Endoplasmic Reticulum – ER

Enhanced Chemiluminescence - ECL

Enterovirus Quantification – EQ

Enterovirus Untranslated Region – EVUTR

Environmental Protection Agency – EPA

Ethylenediaminetetraacetic Acid – EDTA

Exempli Gratia (For Example) – *e.g.*

Expelled Vesicle - EV

Faecal Indicator Bacteria - FIB

Fetal Bovine Serum – FBS

Fluorescin Isothiocyanate – FITC

Free-Living Amoebae – FLA

Genomic Deoxyribonucleic Acid - gDNA

Genomic Equivalent – GE

Gram - g

Gravitational Force Unit – *g*

Guanosine Triphosphate – GTP

Horseradish Peroxidase – HRP

Hours - h

Human Adenovirus – HAdV

Hydrochloric Acid – HCl

Id Est (In Other Words) – *i.e.*

Immunoglobulin - IgG

Internal Ribosomal Entry Site – IRES

International Committee on Taxonomy of Viruses – ICTV

Intracellular Adhesion Molecule 1 – ICAM1

Kilo-bases – kb

Kilodaltons - kDa

Limit of Detection with 95 Percent Confidence – LOD_{95}

Litre - L

Magnesium Sulfate - $MgSO_4$

Microliter - µL

Micromolar Concentration - µM

Microvesicle - MV

Milliamperes – mAmp

Milligram – mg

Millilitre – mL

Millimolar Concentration – mM

Minimum Essential Medium – MEM

Minute – min

Molar Concentration - M

Most Probable Number of Infectious Units per Milliliter – MPN IU/mL

Multiplicity of Infection – MOI

Nanometer - nm

National Center for Biotechnology Information – NCBI

Paraformaldehyde – PFA

Parts per Million – ppm

Peptone-Yeast-Glucose – PYG

Phosphate Buffer Saline – PBS

Phosphate Buffer Saline Tween-20 – PBST

Plaque-Forming Units – PFU

Plasma Membrane - PM

Polymerase Chain Reaction – PCR

Positive-Sense Single-Stranded RNA – (+)ssRNA

Potassium Chloride – KCl

Potassium Dihydrogen Phosphate (Monobasic Potassium Phosphate) - KH_2PO_4

Potential of Hydrogen - pH

Quantitative Microbial Risk Assessment – QMRA

Quantitative PCR – qPCR

Relative Humidity – RH

Reverse Transcription – RT

Ribonucleic Acid – RNA

Ribosomal RNA – rRNA

Roswell Park Memorial Institute – RPMI

Seconds - sec

Serum-Casein-Glucose-Yeast-Extract Medium – SCGYEM

Sodium Chloride – NaCl

Sodium Dodecyl Sulfate – SDS

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis - SDS PAGE

Species (plural) – spp.

Square Centimetre – cm^2

Svedberg Units – S

Tetramethylethylenediamine - TEMED

The Applied Genomics Core – TAGC

Tissue-culture Infectious Dose 50 percent – $TCID_{50}$

Toll-Like Receptor - TLR

Transmission Electron Microscopy – TEM

Tris-Acetate-EDTA – TAE

Type 1 Diabetes – T1D

Type B Coxsackievirus – CVB

Ultra-Violet Radiation – UV

Unit - U

Untranslated Region – UTR

Virion Protein – VP

Volts - V

"Nature is always more subtle, more intricate, more elegant than we are able to imagine."

Carl Sagan, *The Demon-Haunted World: Science as a Candle in the Dark*

Chapter 1: Introduction

1.1 Human enteric viruses and their public health significance

Human enteric viruses, such as enteroviruses, noroviruses, and adenoviruses, are waterborne gastrointestinal pathogens, which are transmitted through the faecal-oral route via contaminated natural, recreational, or processed waters (including drinking water), as well as through the consumption of water-contaminated foods. [1–8] Enteric viruses shed at high titers with the faeces of infected individuals, yet they are infectious at low doses. [9–11] When wastewaters are treated insufficiently and discharged into surface or ground waters, enteric viruses are a leading hazard to human health. [1,9–14] In addition, leaking drinking water pipes can turn into intrusion points during low pressure events in sewage-contaminated soils and groundwaters. [15,16] Enteric virus particles (virions) can persist in both fresh and marine waters for months, often adsorbed to larger organic particles or sediments, but they can also incorporate within water-based biofilms commonly found in plumbing system pipes. [17–20] In some biofilms, the virus titers can become concentrated up to 100-fold, such as in wastewater wetlands, which may then slough off into natural waterways, and subsequently concentrated virus aggregates may enter the water supply system. [20] Virions within biofilms are protected from disinfectants, such as chlorine, ozone, peracetic acid, and even UV. [16,19]

Physical removal of viruses from waters is aided by coagulation, flocculation, sedimentation, and sand media filtration, especially when virions are stabilized on larger organic particles or as aggregates. [10,11,21] Coagulation methods rely on adsorption to coagulant matrices, such as aluminum or $FeCl_3$, followed by rapid sand filtration. [22,23] Such methods depend on the surface charge and isoelectric point of virus capsids, as well as their hydrophobic interactions, all of which could change through environmental stressors, pH fluctuations, variations in ionic strengths from waters, and association with organic particulates. [23–25] Therefore, unpredictable changes in the physical properties of environmental viruses can impair their removal from waters, especially ones associated with organic colloids or as aggregates. [9,23,25–28] In some cases, enhanced coagulation techniques (higher concentration of coagulant and coagulant aids) may be recommended to increase virus removal. [19]

Given the importance of enteric viruses it is surprising that no routine virus detection assays are used in the water industry, nonetheless, for research monitoring enteroviruses and adenoviruses are used as indicators to assess the efficacy of water treatment. [1,9,29,30] Pathogen removal data, along with more intensive faecal indicator bacteria (FIB, *e.g. E. coli* and enterococci) and virus surrogate (*e.g.* MS2 coliphage) data are used to inform likely reference pathogen exposure concentrations (along with their human dose-response relationships) in quantitative microbial risk assessment (QMRA). [16, 31–36] QMRA is used to inform pathogen management of systems, such as for drinking water safety plans, a regulatory requirement in Alberta, Canada and in some 90 other countries worldwide. [37–39] Most drinking water outbreaks in developed regions occur due to lack of understanding of short-term events in systems, hence QMRA is very useful to explore scenarios, such as possible events involving different pathogens during rain, pipe intrusion during pressure changes, water flow biofilm mobilization, sub-optimal water treatment, issues in pathogen detection levels/efficacies, as well as infectious dose variations to humans. [34,40,41] QMRA generally focuses on nominal treatment performance and identifies log-reduction (*i.e.* 90% reduction = 1-$\log_{10}$, 99% = 2-$\log_{10}$ etc.) efficiencies in water treatment stages. [34] Pathogen risks could be associated with a specific type of distribution system, such as intermittent water supply systems, which are more prone to biofilm development, or bathing in a specific recreational water basin, such as urban rivers impacted by outfalls. [42,43] Risk assessment studies are also important for scientific and research development as they can help identify priorities of public health concern. An example high-risk enteric virus group is coxsackieviruses (CV), which belong to the *Enterovirus* genus and which the US Environmental Protection Agency (EPA) has included in their Candidate Contaminant List (CCL). [22,23, 44–46]

1.2 Coxsackievirus epidemiology and significance as water contaminants

1.2.1 Overview

Coxsackievirus epidemiology is dependent on several factors, such as local sanitation systems, seasonality (cases peaking mainly in summer and fall, except for tropical and semi-tropical regions where reports occur year-round), as well as patient susceptibility (particularly small children). [47–52] Outbreaks are linked to geographic area and climate conditions, such as high humidity and

warm temperatures. [50–53] CV outbreaks are problematic for confined and crowded areas, such as kindergartens, day care centres, infant care hospital units, but also open fields such as play grounds. [54–56]

Coxsackieviruses can use multiple receptors to infect host cells, which could happen through multiple routes of exposure (food, water, fomites, aerosols) and lead to a wide range of symptoms. [49,57–63] The virus mainly replicates in the oropharynx and gastrointestinal tract, and sheds in the stool of infected patients for up to several months. [48,64] The most infectious coxsackieviruses are considered to be members of the B subtype (CVB), which have been associated with paralysis, aseptic meningitis, encephalitis, rhomboencephalitis (brain stem infection), febrile illness, acute cerebellum ataxia, cranial nerve palsies, cardiac disease, mucocutaneous infections, myocarditis, pericarditis, pleurodynia, pancreatitis, Type 1 Diabetes (T1D), as well as bronchopneumonia, bronchiolitis, hand-foot and mouth disease, miscarriage, and in genetically predisposed individuals also hepatitis. [47–49,53,54,65–81] Infectious CVB can be transmitted from mother to fetus and this has been shown to affect the neurological development of newborns, in addition to the increased chance of miscarriage. [81–83] Currently there is no available vaccine against CVB and treatment is mainly supportive, although research in the field is active. [84–86] The prognosis of CVB infection is usually good, except for newborns, for whom a matter of days of age could make significant differences for successful recovery. [87–90] Despite the unprecedented host-age specificity of coxsackieviruses in general, members of the B subtype (particularly B2 and B5) have been reported to also infect healthy adults. [75,91] CVB are recognized environmental factors for Type 1 Diabetes. [69,76,92]

1.2.2 Molecular characteristics and susceptibility to disinfection

Coxsackieviruses, like other picornaviruses, are non-enveloped and have a capsid diameter of approximately 25-30 nm. [48,93] The genome is a single-stranded positive-sense RNA molecule, also referred to as "(+)ssRNA", and is comprised of approximately 7,500 nucleotides, roughly 10% of which are non-coding. [94] The capsid is comprised of 12 protein pentamers, build from five protomers of four structural viral proteins (VP-s): VP1, VP2, VP3, and VP4. [48,83,95,96] The capsids are very tightly packed and can withstand highly acidic environments (pH < 3), which is

important for passage through the acidic stomachs of human hosts. [48,97,98] The coxsackievirus virion, like other members of the *Enterovirus* genus, is comprised of approximately 70% protein and 30% RNA and has a sedimentation coefficient of 156 S (Svedberg units). [48] Recommended heat inactivation is 50ºC or higher. [91] CVB reportedly have higher tolerance for chlorine-disinfection as compared to other viruses used for virus-removal studies, including other *Enterovirus* members. [99–102] For at least 4-$\log_{10}$ reduction, CVB requires 15-30 min contact time with a standard dose of 0.2 mg/L of free chlorine, as compared to 1 min requirement for adenoviruses, 5 min for echoviruses, and several seconds for noroviruses. [103] In addition, CVB disinfection can vary depending on the water source, temperature and pH. [103] In some cases, it is recommended to use lower pH for chlorine disinfection, due to the strong oxidizing effect of hypochlorous acid, however reports on pH requirements can be contradictory, especially for viruses resistant to highly acidic environments. [103,104] The high chlorine- and acid- resistance of CVB, together with their diverse routes of entry and pathogenicity, makes them important indicator pathogens for water-treatment evaluation studies and risk assessment, especially as representatives of the *Enterovirus* genus. [47,49,57–61]

1.2.3 CVB taxonomy

Coxsackieviruses were initially described as polio-like viruses, whose taxonomic characterization was very challenging, and as a result, they were named after the city where the first reported cases were observed, in order to avoid delays in clinical research. [47,105] Unlike polioviruses, however, the “Coxsackie viruses” did not infect the central nervous system (CNS) of laboratory mice, but instead caused paralysis due to destructive lesions of the skeletal muscles. [105] The viruses were distinguished due to their unprecedented host-age preference. [105,106] Two virus groups were initially reported - “A” and “B” respectively, of which type B coxsackieviruses were more infectious and difficult to isolate. [47] In subsequent years, the International Committee on Taxonomy of Viruses (ICTV) assigned group A coxsackieviruses into the *Enterovirus A* and *C* species, while group B coxsackieviruses were classified as *Enterovirus B* species of the genus *Enterovirus*, family *Picornaviridae*, order *Picornavirales.* [107,108] Despite different clinical presentations, type B coxsackieviruses have identical genome organization and virion size as

polioviruses (*Enterovirus* C), which are often cross-referenced on reports of life cycle and replication (Figure 1.1). [109,110]

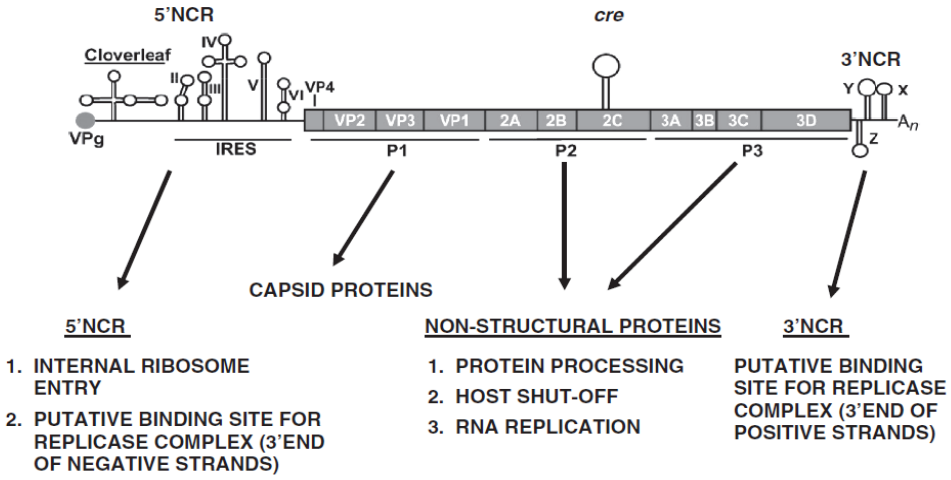


Figure 1.1 Genome organization of coxsackievirus B3 (obtained from Sean and Semler, 2008) [94]

1.2.4 CVB infectious cycle and replication

The major receptors and co-receptors used by CVB for cell attachment are the Coxsackie-Adenovirus Receptor (CAR), Intracellular Adhesion Molecule 1 (ICAM-1), as well as some integrins, Decay Accelerating Factor (DAF), occludin, and heparin sulfate. [57–61,111] ICAM-s are glycoproteins expressed on epithelial cells, endothelial cells, and leukocytes. [112] CAR is expressed on all epithelial cells as a transmembrane component of the tight junctions and regulator of E-Cadherin homeostasis, but also on cardiac cells, where it is involved in the regulation of cell-cell communications. [113–118] CVB entry into polarized epithelial cells requires DAF on the apical surface together with occludin (another tight-junction component), as well as GTPases and kinases involved in micropinocytosis. [119,120] CVB binding to DAF triggers cellular signals and DAF clustering (Figure 1.2), followed by binding to CAR and entry through caveolin-dependent endocytosis. [96,120] In some cases, CVB entry involves calcium signaling, which is cell-line specific. [121] CAR expression is tightly regulated in the brain, heart, CNS, developing heart, lung epithelia, polarized epithelia, pancreas, kidneys, and its expression is especially heightened during tissue development, therefore explaining the characteristic coxsackievirus host-age preference. [122,123]

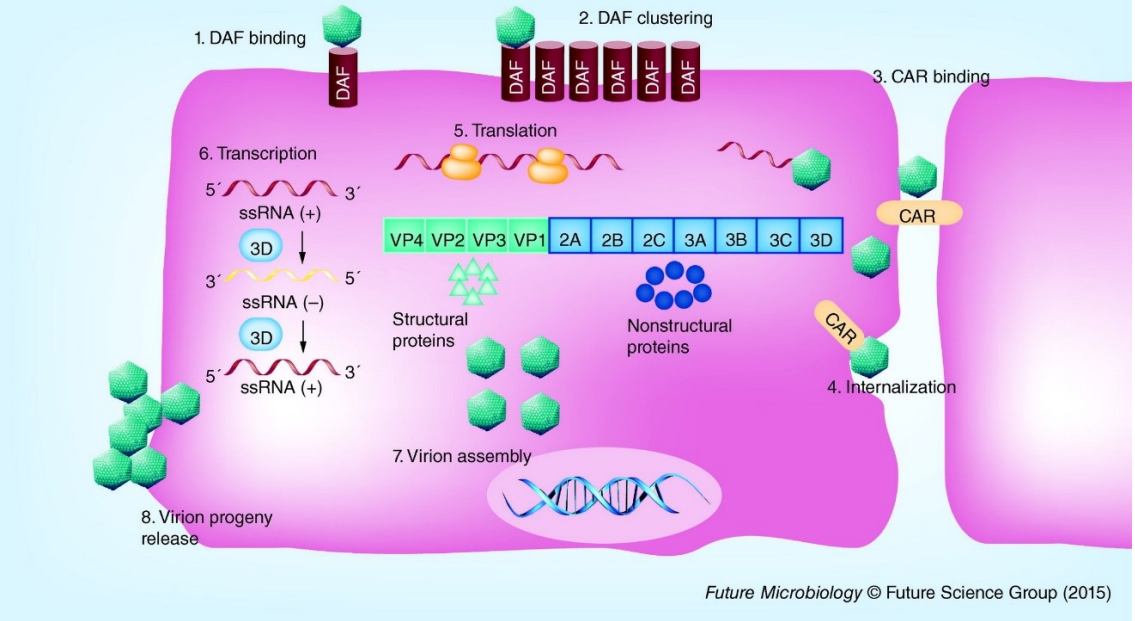


Figure 1.2 Coxsackievirus B3 life-cycle (obtained from Garmaroudi *et al.,* 2015) [96]

Following cell-receptor binding, internalized virions are trafficked to the endosomes, where they partially un-coat, forming intermediate structures called "A-particle"-s. [124] This allows the N-termini of VP1 proteins to protrude from the capsid and stabilize the virion into endosomal membranes, which the virus can later permeate with the help of pores comprised of VP4 subunits. [110,119] For polioviruses and type B coxsackieviruses, unlike other members of the *Picornaviridae* family, the release of the genome from the A-particle is delayed for up to 90 min until currently unknown factors trigger viral RNA release into the cytoplasm. [109,110] Virus genome replication can begin as soon as host ribosomes bind to the 5' UTR (untranslated region), which acts as an internal ribosomal entry site (IRES) on the viral genome. [48,93,109,125] In addition, a *cis*-acting replication element on 2C (*cre*) is involved in uridylation of VPg, which serves as the primer for the RNA-dependent RNA polymerase 3D. [96,109,126] CVB replication depends on the formation of double-membrane vesicles, which serve as virus replication factories. [127,128] Replication factories may reach 650 nm in length by 80 nm wide, but their size can vary throughout the stages of infection. [128] During the formation of these membranous vesicles cholesterol molecules are targeted from the host plasma membrane to the viral replication factory. [84,129] The 2B protein aids in the process by modifying endoplasmic reticulum (ER) and plasma membrane proteins of infected cells. [94,130]

The first replication step is the synthesis of negative-sense single-stranded RNA, which is then used as template for the synthesis of multiple (+)ssRNA molecules. [122] For the virus to utilize the same strand for RNA synthesis and translation the 3D polymerase moves from the 3'- to 5'- end on the viral genome, in the opposite direction of the ribosome. Enterovirus RNA undergoes cap-independent translation, which is in contradiction with the host-cell requirements for cap-dependent translation and therefore, up to two hours post-infection the viral proteases 2A and 3C begin to interfere with host transcription and translation machineries. [93,109,131] The protease 3C blocks transcription, while 2A cleaves host translation initiation factors. [132] In addition, a secondary stem-loop element also interacts with host proteins, while viral proteases are mainly involved in processing the newly synthesized polyprotein. [48,65,109,131,133] Coxsackieviruses, like all enteroviruses, are capable of rapid evolution and adaptation due to their high mutation rates (0.1 to 3 mutations per replication event), which could lead to multiple determinants of virulence and tropism, but also to lethal mutations (error catastrophe). [49,85,111,133,134] CVB has also been reported to utilize the host autophagosome machinery to infect new cells. [96] The autophagosomes are double-lipid membrane vesicles, which engulf macromolecules and are involved in eukaryotic protein degradation by fusing with acidic lysosomes. [96] CVB3 has been shown to induce the formation of acidic autophagosomes, prevent fusion with lysosomes, and replicate within "hijacked" autophagosomes, as well as exit infected cells through them. [129,135–140] In addition, the virus can cause the phagosomes to merge and form even larger vesicles called megaphagosomes. [129,137] CVB can also exit host cells through apoptic microvesicles (Figure 1.3), thus utilizing both apoptosis and autophagy throughout its infectious cycle. [139,141]

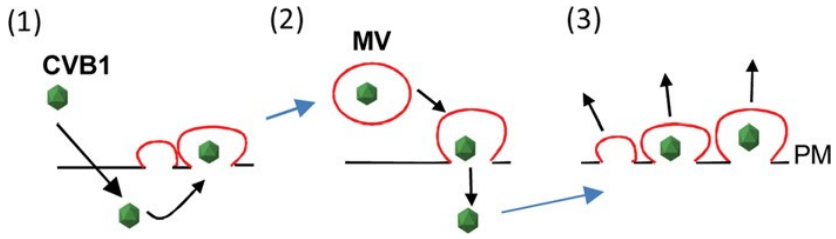


Figure 1.3 Coxsackievirus B1 transmission through apoptic microvesicles (MV). MV-s can aid the virus infection by fusing with the plasma membrane (PM). (obtained from Inal and Jorfi, 2013) [141]

Despite being highly lytic, CVB also causes latent infections (T1D, dilated cardiomyopathy, infections of the thymus and pancreas) and becomes actively replicating and lytic again as soon as the infected cells become activated. [63,95,142,143] The first line of immune defense against CVB is innate immunity, mainly through Toll-Like Receptors (TLR) or RNA helicases, which can recognize double-stranded RNA. [144–146] TLR3 recognition by macrophages, as well as their nitric oxide production, is essential for clearing the initial CVB infection, even though some CVB have been shown to productively infect macrophages, especially the monocytes of T1D patients. [92,147,148] Monocytes are the main trigger for myocardial tissue damage, causing monocytosis, which is especially observed in the presence of Coxsackievirus infections. [149–151] CVB persistence inside migratory immune cells has been described as a possible "Trojan horse"-like scenario for virus dispersion and entry across epithelial tight junctions. [84,92,147,152,153]

1.2.5 CVB importance in the context of complex microbial interactions, environmental persistence, and risk assessments

CVB provide an excellent model for studying virus involvement in complex microbial interactions, which could occur in water-based biofilms, such as the poorly understood virus-amoebae interactions. The complexity of biofilm communities is significantly shaped by natural predators and parasites, such as free-living amoebae (FLA) and bacteriophages. FLA are eukaryotic phagotrophs, which graze on water-based biofilms and are present throughout all stages of in-premise water distribution (Figure 1.4). [154–156] CVB can infect eukaryotic phagocytes, such as macrophages, utilizing them in a proposed "Trojan horse"-model of dispersion across epithelia, and in addition the virus can resist acidic environments and so may evade digestion within acidic vacuoles and utilize other vesicles throughout its infectious cycle. [129,135–141,147,148] While bacteriophages would be expected to be digested, CVB are likely to withstand exposure to FLA in environmental waters and especially in biofilms, where proteinaceous enteric viruses tend to incorporate. [157] In addition, the epidemiology and pathogenicity of CVB make them relevant research priorities in risk assessment studies, where complex factors involving virus persistence and dispersion can be important knowledge gaps. [10,31,45] For example, the complex interactions between FLA and human pathogens, such as bacteria, could lead to the persistence of amoeba-resistant bacteria within FLA, where the bacteria could gain shelter and means of dispersion

(Figure 1.4). FLA interactions with phagocytosed pathogens significantly complicate the risk assessment process and water safety management, as studies rely on comprehensive data sets involving multiple variables, such as pathogen concentration, resistance to disinfection, detection, removal, and even selection for phagocyte resistance. While FLA-bacteria interactions are well-researched, currently FLA-virus interactions are poorly understood, which is a public health concern when it comes to high-risk contaminants, such as *Enterovirus* members, and particularly the highly infectious CVB, which are likely to co-exist with FLA species ubiquitous in waters, including tap water. [154,158]

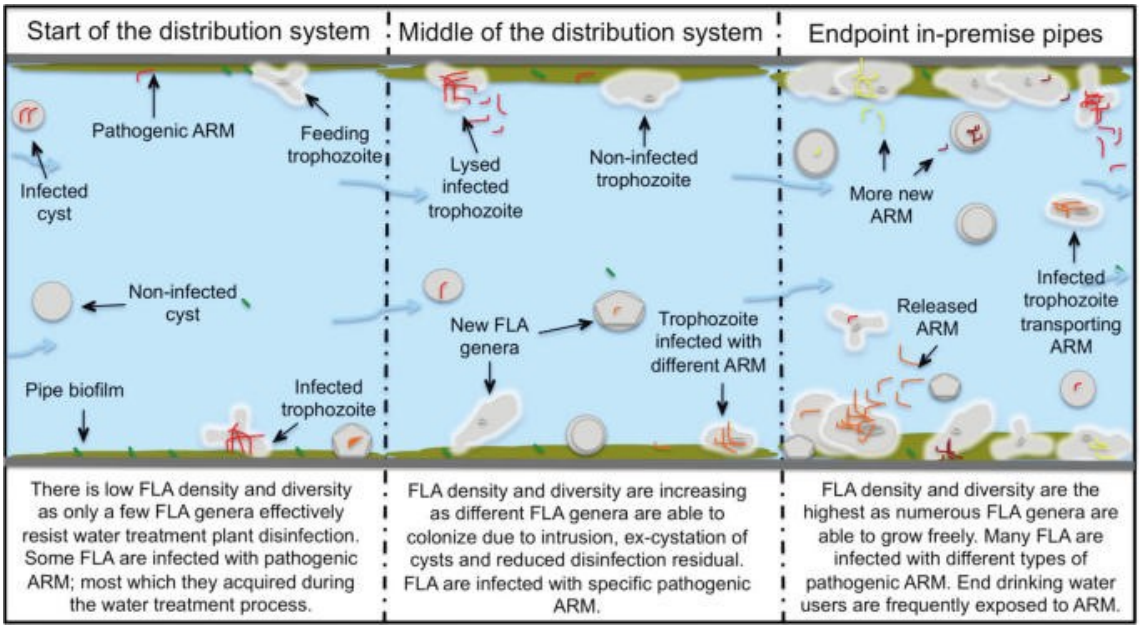


Figure 1.4 Schematic diagram of possible free-living amoebae (FLA) interactions with amoeba-resistant microorganisms (ARM) at different stages of water distribution (obtained from Thomas and Ashbolt, 2011) [154]

1.3 Free-living amoebae

1.3.1 Overview

Free-living amoebae (FLA) are non-parasitic environmental phagocytic protozoa. [159,160] They inhabit natural waters, soil, air, compost, sediments, engineered systems, such as water treatment plants, drinking water taps, and cooling towers, where they feed on bacteria, fungi, algae, and other

protozoa. [161–166] FLA play an important role in controlling microbial populations in the aquatic systems and soils, as well as in phosphorus and nitrogen turnover. [160,163,167,168] The main amoebae associated with human disease are *Acanthamoeba* spp., *Balamuthia mandrillaris* and *Naegleria fowleri*, all of which are associated with fatal brain pathologies. [168,169] *Acanthamoeba* spp. also cause corneal keratitis and encephalitis and are particularly harmful to immunocompromised individuals, transplant patients, and contact lens wearers. [160]

The most common FLA genera isolated from in-premise water systems are *Vermamoeba* and *Acanthamoeba.* [163,170–174] Both amoebae have two life stages – an actively feeding motile trophozoite form, and a dormant cyst, which forms under various environmental stress conditions (Figure 1.5) and quorum-sensing ques. [173,175] Trophozoites attach to surfaces, where they feed by receptor-mediated phagocytosis, acid-dependent phagocytosis, or pinocytosis. [176–178] Food particles are internalized in digestive vacuoles, the fate of which depends on the nature of the food, as in some cases undigested material is expelled in vesicles to the surrounding liquid environment. [179] At increasing cell densities, amoebae can emit pheromones and pheromone-like molecules to signal to neighboring FLA, and therefore affect one another's encystment. [161,180–182] FLA cysts are very resistant to chlorination, UV treatment, heat shock, organic chemicals, and to some extent to hydrogen peroxide. [163,169,170,173,183–185]

1.3.2 *Vermamoeba vermiformis*

Vermamoeba vermiformis, formerly known as *Hartmannella vermiformis*, is the only species of *Vermamoeba* currently identified in drinking water systems, but was first described in 1967, based on its elongated motile form, although it has a round shape when suspended in liquid. [176,186] Current FLA classification is based on pseudopod structure, replication patterns, as well as 18S rRNA gene typing. In 2011 the genus *Vermamoeba* was created to accommodate the formerly named *H. vermiformis.* [186–188] *V. vermiformis* belongs to the supergroup Amoebozoa -> clade Tevosa -> subclade Tubulinea -> Echinamoebidia -> family *Vermamoebidae* -> genus *Vermamoeba* -> spp. *Vermamoeba vermiformis*. [189] When actively feeding, *V. vermiformis* can form two or more pseudopods. [188] It has a crescent shape when turning, and a cylindrical shape when moving straight. [186] The amoeba has 1- 4 contractile vacuoles (CV) present at a time, which

transport ions between the cytoplasm and the liquid environment for osmotic balance. [186,190] Various conditions, such as lack of nutrients, osmotic pressure, temperature changes, pH changes or lack of space for growth trigger encystment. [160] Encysting *V. vermiformis* cells have refractive granules and their cysts are smooth and circular (Figure 1.5). [186] Upon favorable conditions, *V. vermiformis* excyst by softening the cyst wall and transitioning back to a trophozoite form, leaving the empty cyst wall behind or partially digesting it. [35,170]

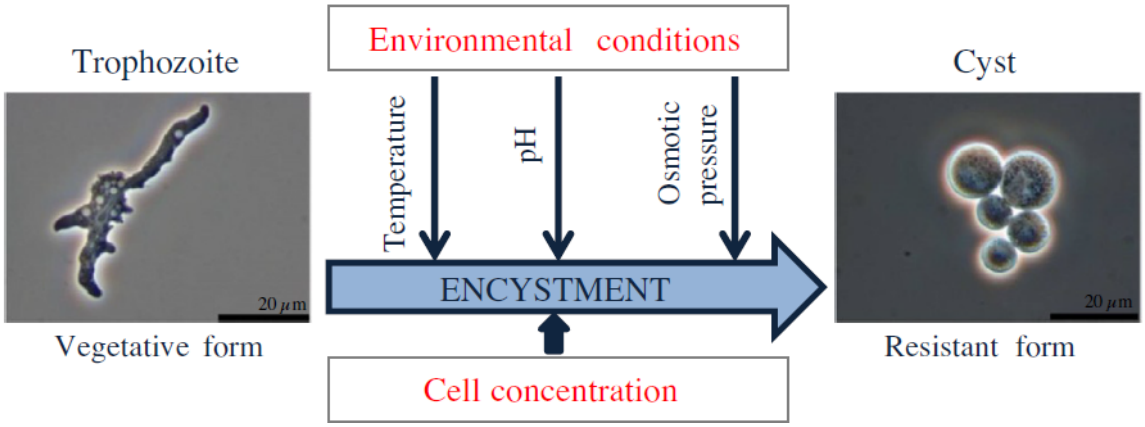


Figure 1.5 *V. vermiformis* life stages (obtained from Fouque *et al.,* 2014) [161]

Shortly after they excyst the amoebae are metabolically active and have large digestive vacuoles filled with lysosomes and partially digested material, as well as large number of autophagosomes and contractile vacuoles. [170]

V. vermiformis can survive in hot water tanks due to its ability to withstand prolonged exposure to ≤ 55ºC. [174] In addition, the cysts can withstand 4 ppm (parts per million) of free chlorine, which is higher than the 0.75 - 1.5 ppm often used for water disinfection. [191] *V. vermiformis* cysts are also resistant to detergents, such as SDS (sodium dodecyl sulfate) and reportedly many brand-name biocides used in dental water line units (ICX, Sterilox, Alpron, Oxygenal 6, Dentosept), in addition to bromine and isothiazolinone used in cooling towers. [161,184,192,193]

The main habitat for *V. vermiformis* is freshwater and it is currently the most commonly reported FLA species in industrial water distribution systems, followed by *Acanthamoeba* spp. [170,174,186,191,194–197] *V. vermiformis* is also an environmental reservoir for the persistence and

dispersion of the respiratory pathogenic bacterium *Legionella pneumophila* of in-premise water systems, such as drinking water, showers, and dental waterline units, which the amoeba can colonize to a significant degree after only 24 h of stagnation. [155,184,191,198–200] Even though *V. vermiformis* is not considered to be a major human pathogen, due to its high prevalence in tap water, it has been reported to cause keratitis in some contact lens wearers, as do *Acanthamoeba* spp. [201,202] In some cases, undescribed *Vermamoeba* species have been associated with meningoencephalitis and bronchopneumonia. [203]

Overall, *V. vermiformis* is assumed to be an important agent for nosocomial infections, due to its presence in hot water systems of hospitals, where it is the most dominant FLA species, compared to moist surfaces where *Acanthamoeba* and *Naegleria* spp. are also very common. [174,204–206] *V. vermiformis* strains isolated from moist surfaces and from hot water tanks differ in heat tolerance, presumably due to the adaptability of the amoeba, which can also thrive in cold drinking waters. [174,194,207]

1.3.3 *Acanthamoeba polyphaga*

Acanthamoeba polyphaga belongs to the supergroup Amoebozoa -> clade Tevosa – subclade Discosea -> Centramoebia -> family *Acanthamoebidae* -> genus *Acanthamoeba* -> spp. *Acanthamoeba polyphaga*. [189] The *Acanthamoeba* genus has 17 genotypes, of which the one associated with human infections is mainly T4, including the species *A. polyphaga.* [160] The name of the genus is derived from the Greek word “acanth” meaning “spike”, which relates to unique *Acanthamoeba* spp. pseudopod-like pointed structures called “acanthopodia” (Figure 1.6). [160,176] Most *Acanthamoeba* spp. are considered opportunistic human pathogens, which cause cutaneous lesions, sinusitis, keratitis, but can also cross the blood-brain barrier and cause fatal encephalitis or in rare cases granulomatous brain tumors. [160,176,208–212] *Acanthamoeba* spp. infections are especially concerning for immunocompromised patients and contact lens wearers. [160] The amoebae reportedly show signs of neuraminidase activity, which could be a factor in keratitis infections, while some species and strains have also been shown to express homologues of DAF and therefore interfere with the host complement. [213] The amoeba also has ecto-ATPases involved

in cytopathic effects on monocytes and other cells. [214,215] In addition, its proteases can degrade host immunoglobulins, cytokines, and complement proteins. [213,216]

Acanthamoeba spp., such as *A. polyphaga* and *A. castellanii*, have been isolated from diverse environments, including: sea water, ocean sediments, beaches, ponds, soil, fresh water lakes, hot springs, salt water lakes, Antarctica, water-air interfaces, air conditioning units, bottled water, distilled water, cooling towers, electrical and nuclear power plants, hot tubs, ventilation ducts, sewage, compost, shower heads, kitchen utensils, drinking fountains, eye wash stations, humidifiers, vegetables, surgical instruments, dialysis machines, contact lenses, as well as in association with healthy animals, fresh water fish, and even healthy humans (in the throat, lungs, nasal cavities, stool, sinuses, and urine). [160,176,209,210,217–222] Hence, *Acanthamoeba* spp. are considered environmentally ubiquitous.

A. polyphaga mainly replicates by binary fission, has pointed spindles during mitosis, and its cysts have characteristic double walls (Figure 1.6). [160,176,223] It moves equally well on solid and moist surfaces and forms strong adhesion forces on liquid-air interfaces. [160] *A. polyphaga* can have several contractile vacuoles, digestive vacuoles, lysosomes, and a great number of glycogen-containing vacuoles. [160] The plasma membrane consists of proteins, phospholipids, sterols and lipophosphonoglycans. [160,224,225] The nucleus is about one sixth the size of the trophozoite, however multiple nuclei could also be present at a time. [160] The trophozoite and cyst have similar sizes of about 13-23 μm, but cysts are usually smaller. [160] The optimal growth conditions are ambient temperature, food supply and neutral pH. [160] The encystment process starts by the expulsion of excess water, food particles and organic particles, followed by shrinking and the formation of a pre-cyst intermediate. [176] The cysts have minimal metabolic activity. [160] The inner wall is comprised of cellulose, while the outer wall of proteins and polysaccharides, including ostioles, which sample the surrounding environment for nutrients and favorable conditions. [160,176,226–228] Cellular levels of proteins, RNA, triglycerides and glycogen within the cysts are generally very low, and even the nuclear volume is decreased, together with the Golgi network and mitochondrial volumes. [176,229] Both encystment and excystment, however, require active synthesis of macromolecules. [176]

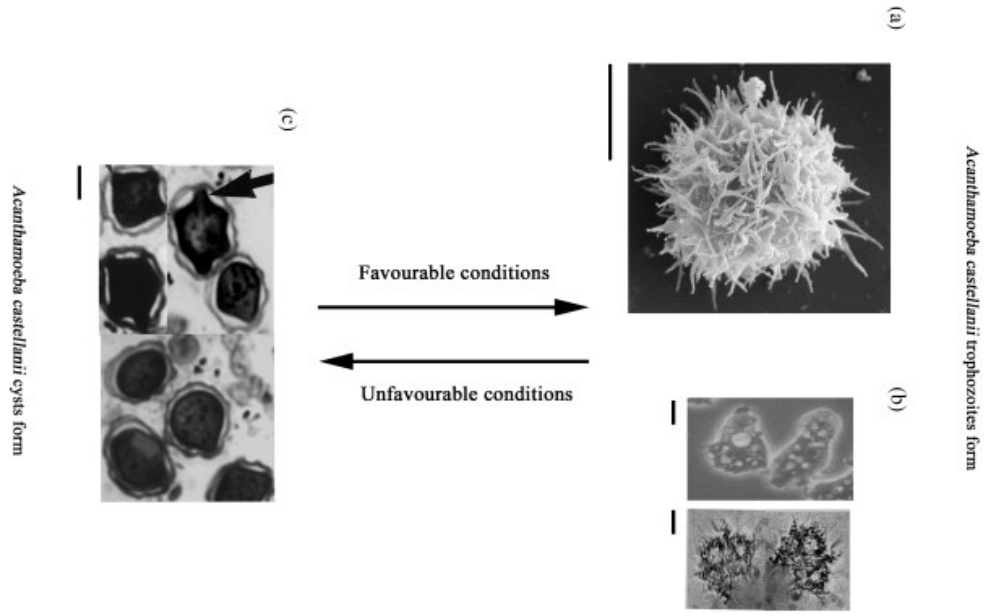


Figure 1.6 *A. castellanii* life stages. A representative member of the *Acanthamoeba* genus - trophozoites observed with scanning electron microscopy (a), and phase contrast microscopy (b), and cysts observed with phase contrast microscopy (c) (obtained from Khan, 2006) [176]

The major problem in combating *Acanthamoeba* spp. persistence, is their reported tendency to encyst very rapidly in response to a wide range of chemical agents. [212,230,231] Like *Vermamoeba* spp., *Acanthamoeba* spp. cysts are also resistant to chlorine and heat and can persist in the environment for years. [232–234]

1.3.4 Free-living amoebae as reservoirs for the environmental survival and dispersion of human pathogenic bacteria

In engineered water systems, including drinking water and dental waterline units, bacteria often form biofilms, which are usually not a health hazard, however, they could attract diverse microorganisms, depending on biofilm composition, pipe material, pipe corrosion by-products, and even type of disinfectants used in the system. [168,184,199,200,235] FLA can influence the physical characteristics of such biofilms by affecting bacterial metabolism and nutrient turnover. [156,167,236,237] Some pathogenic bacteria, such as *Pseudomonas aeruginosa* are well adapted to FLA colonization of their biofilms and can even infect the amoebae, leading to a link to some nosocomial infections. [238–241] In addition, since the early 90-s, *V. vermiformis* is consistently

reported in association with *Legionella pneumophila,* especially in hospital plumbing systems and cooling towers. [36] Internalized bacterial pathogens are protected from chlorine and can persist in drinking tap water together with their FLA hosts. [194,207] *V. vermiformis* can engage in specific molecular interactions with internalized *L. pneumophila*, which can in turn prime its virulence and adapt to replication inside other phagocytes, such as macrophages. [242–246] Fresh water amoebae, such as *V. vermiformis* and *Acanthamoeba* spp., are considered to be the primary environmental reservoirs for *L. pneumophila* persistence and dispersion from water distribution systems, such as via devices that generate aerosols (showers, tap aerators, humidifiers etc.). [154,155,194,195,247,248]

FLA are also reported to harbor other pathogens, such as *Neochlamydia hartmannellae*, *Klebsiella pneumoniae, Francisella* spp. (causing tularemia), non-tuberculous *Mycobacterium* spp., *Stenotrophomonas maltophila* (associated with catheters and ventilation tubes), *Salmonella enterica, Listeria monocytogenes*, *Campylobacter jejuni*, *Vibrio cholerae,* toxigenic *E. coli* as well as *Shigella* spp. [159,185,249–260] *S. enterica* can replicate in the contractile vacuoles of *A. polyphaga,* while *L. pneumophila* grows within modified digestion vesicles, which lyse and allow full intracellular growth, or gets expelled in vesicles and remain protected from antibiotics. [256] Various pathogenic bacteria can persist in high concentrations in expelled FLA vesicles. [154,261] Opportunistic fungal pathogens, such as *Candida* spp. and *Aspergillus fumigatus*, can also persist inside *V. vermiformis* in dental units and hospital water systems. [193,262–264] The ability of FLA to harbor phagocyte-resistant human pathogens, which can replicate in the amoebae, seek shelter from disinfection, and be expelled in high concentration through expelled vesicles, presents multiple health risks, and can affect bacterial density in the air, water, or biofilms, as well as exposure to human respiratory tracts. [154,155,261,265] All aspects of such complex microbial interactions need to be considered for QMRA to better evaluate and improve existing water treatment practices, and this involves important missing links, such as the association of FLA with human enteric viruses. [155,261]

1.4 The "missing link" in microbial risk assessments – amoebae as reservoirs for human enteric viruses

Currently FLA-virus interactions are not well understood. *Acanthamoeba* spp. have been found in association with some giant viruses, such as the putative respiratory virus Mimivirus, also known as *Acanthamoeba polyphaga* Mimivirus (APMV). [160,176,266–268] Other giant viruses found in amoebae include *Pandoravirus*, *Marsiellevirus*, and *Faustovirus.* [256,269–272] Giant viruses are not considered human pathogens, and mainly infect algae, protozoa, sponges, corals, and zooplankton. [268,273–277] APMV can infect *A. polyphaga* by phagocytosis, however cysts are resistant to infection. [277,278] APMV can be internalized in macrophages, but its ability to infect them is not well understood. [158,278] The discovery of giant viruses in FLA raised the question of whether FLA could also be vectors for the environmental persistence and dispersion of human pathogenic viruses. [276,279]

The first reported suspicion of human virus in association with amoebae was in 1948, when several clinicians suspected that larger microorganisms in sewage, such as *Amoeba proteus*, could be carriers of polioviruses. [280] The Lansing strain of poliovirus was found to be ingested by the amoeba, but it also caused disintegration of the FLA and did not persist inside. [280] In 1963 hamster-adapted poliovirus strains were tested for persistence in *A. castellanii* by measuring virus infectious titer as tissue-culture infectious dose 50% ($TCID_{50}$) which significantly declined as result of exposure to the amoeba. [281] Another report described the co-occurrence of Herpes Simplex Virus and *Acanthamoeba* keratitis in a patient, however virus-amoebae association was not suspected. [282] In 1981 vaccine strains of poliovirus and some echovirus serotypes were shown to adsorb to the surface of *A. castellanii*, but without any evidence for internalization. [283] The topic was largely unexplored until recent years when electron microscopy and fluorescence microscopy images revealed Human Adenovirus (HAdV) types 11 and 41 internalized in *A. castellanii*, however, evidence of viral replication or infectivity were not provided. [284] HAdV types 1, 2, 5, 8, and 37 reportedly co-occur with *Acanthamoeba* spp. in recreational waters (beaches and swimming pools), but without evidence for internalization in the amoebae. [285–287] *A. polyphaga* has been shown to internalize HAdV and protect it from chlorine, however, internalized virion persistence over time appears unreported. [287] In addition, *Norovirus* surrogates

(Murine Norovirus Type 1) have been shown to adhere to the surface of *A. castellanii* and *A. polyphaga*, get internalized by the amoebae, and persist as plaque forming units (PFU/mL). [288] Coxsackievirus B3 has been reported to persist in *A. castellanii* as infectious $TCID_{50}$ titer over time, together with trypsinization studies, which demonstrated that the virus could adsorb to the surface of the amoebae, however, conclusive data on virus internalization is missing. [289] Other studies report that *A. castellanii* did not internalize coxsackievirus B3, rotavirus Wa, and poliovirus type 2 strains suspended in liquid, but only through grazing on infected mammalian cells. [290,291] In the latter reports, however, co-cultures were performed in cell-culture media, which is not optimal for FLA growth and would not allow for proper judgment on their ability to feed through pinocytosis in general. In addition, some studies measured virus persistence by non-quantitative conventional polymerase chase reaction (PCR), while others checked for infectious titer only, therefore reasons for the contradictory findings are difficult to conclude. In addition, some of the reports include extensive washing steps on *Acanthamoeba* spp. which encyst very rapidly, however the possibility of virus loss through expelled amoebae vesicles is not addressed. [291] In addition, FLA-bacteria interaction could be highly specific, depending on the species involved, and therefore FLA-virus interactions may also be quite diverse. [242–246,292] It is also important to consider the choice of strains for such pioneering assays, such as clinically relevant virus isolates as opposed to environmentally challenged or lab-adapted strains.

1.5 Research aims and objectives

The principal question being addressed in this thesis is: Could an infectious clinical isolate of coxsackievirus B5 be internalized by the free-living amoebae *Vermamoeba vermiformis* (isolated from a hospital cooling tower), or by the environmentally ubiquitous *Acanthamoeba polyphaga* (isolated from a human corneal scraping)? Internalized virions could resist phagocytosis and persist in the amoebae over time in terms of virus copy number and/or infectious titer.

Therefore, the aims of this thesis were to investigate FLA-virus association by using an infectious clinical virus isolate of high-risk status (as opposed to environmentally challenged or lab-adapted strains), in association with amoebae which are either commonly reported in drinking water systems, including hot water tanks, dental waterline units, and especially hospital water networks

(such as *V. vermiformis*), or environmentally ubiquitous in waters and a wide range of moist surfaces (such as *A. polyphaga*). Since viruses are dependent on their hosts, another important aspect of this research is the investigation of FLA-virus interactions using not only ubiquitous FLA species, but also highly evolutionary diverse ones. The major goal is to investigate this interaction in co-cultures performed at optimal conditions for FLA metabolism and active feeding, while investigating virus internalization and persistence by combinations of methods, as follows:

1. Virus persistence as infectious titer and/or quantified genomic equivalents was assessed through a combination of infectivity assays (calculated by independent formulae) as well as qPCR, while also tracking possible cytopathic effects on the amoebae;
2. Virus internalization was assessed with transmission electron microscopy and/or fluorescence microscopy;
3. Long-term virus persistence was assessed for virions co-cultured with *A. polyphaga* 20 months after their introduction, in particular, for association with mature long-term surviving *A. polyphaga* cysts.

Overall these aims addressed knowledge gaps in the association of FLA with infectious human enteric viruses (Figure 1.7), while also providing a research methodology, which could be applied to the investigation of other FLA-virus interactions (involving other species), which may be challenging due to the diverse nature of the organisms involved.

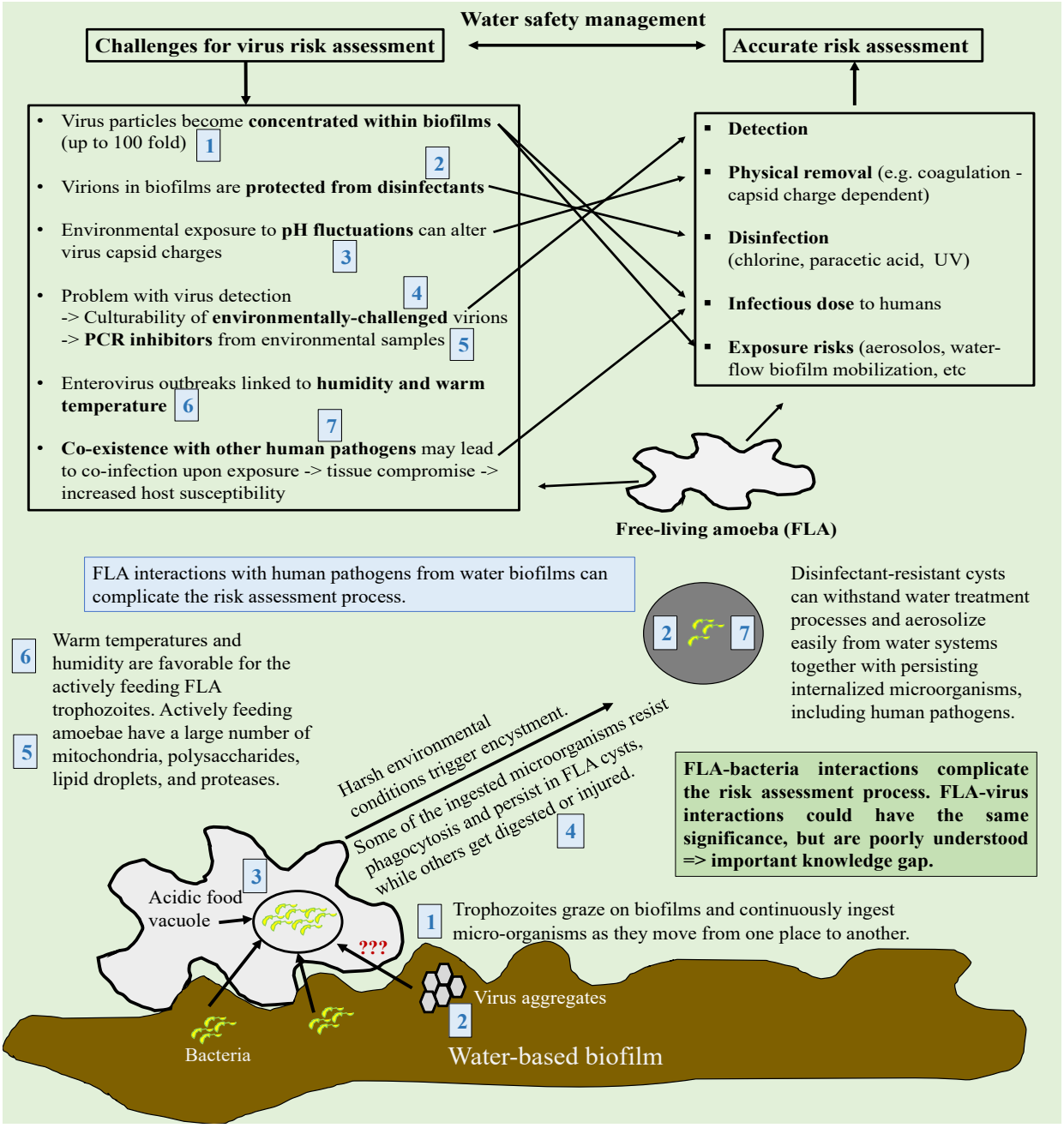


Figure 1.7 Conceptual model highlighting important knowledge gaps involving free-living amoebae (FLA) that may impact current microbial risk assessment of waterborne viruses.

Chapter 2: Materials and Methods

2.1 Identity of the virus isolate

A clinical *Enterovirus B* isolate was provided by Dr. Xiao-Li Pang at Provincial Laboratory for Public Health (ProvLab), Alberta Health Services (AHS), Edmonton. Before the virus was received for the current study, it had been passaged several times *in vitro* by ProvLab personnel and the provided virus suspension therefore contained no biological patient material as confirmed by ProvLab staff. Details about the virus isolation and patient information were purposely omitted to comply with AHS regulations. The identity of the isolate was confirmed as coxsackievirus B5 (CVB5) Faulkner strain by sequencing the 5'Untranslated Region (5'UTR) of the genome with previously described primers, here referred to as EVUTR1 (forward 5-CCT-TGT-GCG-CCT-GTT-TT-3) and EVUTR2 (reverse 5-ATT-GTC-ACC-ATAAGC-AGC-C-3). [293] The primers were chosen as an alternative to the use of VP1-specific primer panels for the purpose of faster genotyping. The EVUTR primers target a big portion of the 5'UTR of the *Enterovirus* genome, which is in proximity to the VP1 region, and which has been reported as prone to high recombination rates among enteroviruses, therefore being a good target for serotyping. [293]

To prepare the virus 5'UTR for sequencing total RNA was extracted from a cell-free virus suspension, using MegaZorb RNA extraction kit (Promega MB2004) with a protocol modified from the one provided by Dr. Xiao-Li Pang (Figure 2.1). Extracted RNA was then subject to reverse-transcription (RT) using SuperScript II reverse transcriptase (Figure 2.1). Freshly prepared viral complementary DNA (cDNA) was then used for conventional Polymerase-Chain Reaction (PCR) with the primers EVUTR1 and EVUTR2 to amplify the 5'UTR target sequence. The PCR cycle consisted of initial denaturation at 95ºC for 3 min, followed by 40 cycles of [denaturation (95ºC for 30 sec), annealing (54ºC for 30 sec), and extension (72ºC for 40 sec)], after which a final extension step was included at 72ºC for 7 min. The size of the double-stranded DNA (dsDNA) PCR product was compared to Gene Ruler 1kb Plus DNA Ladder (Thermo Fisher SM1333) on 2% agarose gel prepared with ultra-pure agarose (Thermo Fisher 16500500) in 1x TAE (Tris-Acetate-EDTA) buffer, comprised of 40 mM Trizma Base (Tris; Sigma T1503), 20 mM Acetic acid (Thermo Fisher 351270-212), 1 mM Ethylenediaminetetraacetic acid (EDTA) disodium salt

(Thermo Fisher BP120-1) in distilled water. The gel was stained with SYBR Safe DNA gel stain (Invitrogen S33102) and was run at 100 V and 400 mAmp for 35 min in a Fisher Biotech electrophoresis unit. The PCR product was visualized with a transilluminator, excised from the gel, and purified with a QIAquick gel extraction kit (Qiagen 28704). The concentration of the extracted dsDNA was measured in triplicates using a Qubit 3.0 Fluorometer (Thermo Fisher Q33216) following the provided manufacturer instructions, after which 7.36 ng dsDNA were submitted for Sanger Sequencing at The Applied Genomics Core (TAGC) at the University of Alberta. The forward and reverse sequencing reactions were supplied with 3.2 μM of EVUTR1 or EVUTR2 primers respectively. The sequencing results were processed with FinchTV software, where the quality of the nucleotide-specific peaks was analyzed, and misidentified nucleotides corrected. The forward and reverse reactions were aligned using the National Center for Biotechnology Information (NCBI) online alignment tool to identify the overlapping portion (contig) among the forward and reverse sequencing reactions. In the case of a mismatch, the peaks were re-analyzed, and their quality was compared to identify the correct nucleotide. The contig sequence and the full 510bp (base pair) sequence were both compared to existing published virus genome databases with the NCBI online Megablast tool, in order to confirm the highest similarity to published *Enterovirus* genome databases.

Flow Chart: RNA Extraction and Reverse Transcription

a) **RNA Extraction:**

- Mix 20 µL of kit-included proteinase K with 200 µL Lysis Buffer and 200 µL sample in a microfuge tube.
- Vortex and Incubate at 56°C for 10 minutes.
- Add 500 µL Binding Buffer and 20 µL MegaZorb reagent (magnetic beads).
- Incubate at room temperature for 10 min. manually mixing every 2 minutes.
- Sediment particles on magnetic rack, aspirate supernatant and wash twice with 1 ml of the provided Wash Buffer.
- Re-suspend washed beads into 50 µL nuclease-free water, mix gently and incubate for 10 min. at room temp. to dissociate MegaZorb-bound RNA.
- Sediment magnetic beads and transfer the supernatant to a clean microfuge tube.
- Use fresh RNA immediately for reverse transcription.

b) Reverse-Transcription (RT):

- Prepare RT master mix per reaction as follows:

Reagent	Volume per reaction	Final concentration
5x First-Strand Buffer (provided with enzyme)	2 µl	1.33 x
Dithiotreitol (DTT; provided with enzyme)	0.5 µl	6.6 mM
Deoxynucleotides (dNTP-s; Invitrogen 10297-018) [2.5 mM each]	1.5 µl	0.5 mM each
RandomPrimers (Invitrogen 48190-011) [300 ng/µL]	1 µl	300ng
RNaseOut (Invitrogen 10777-019) [40 U/µL]	0.25 µl	10U
SuperScript II (Invitrogen 18064-014) [200 U/µL]	0.25 µl	50U
Nuclease-Free Water (Invitrogen Ambion AM9937)	2 µl	
Total	7.5 µl per reaction	
RNA Template	2.5 µL	

Incubate RT reactions at 42°C for 1 hr, then inactivate the transcriptase at 70°C for 15 minutes.

Figure 2.1 Flow chart of RNA extraction and reverse transcription procedures. The protocol was modified from that described by Dr. Pang (ProvLab) to accommodate for smaller reaction volumes. RT reactions, like conventional PCR, were performed on a Nexus Gradient Mastercycler (Eppendorf 6331).

2.2 CVB5 propagation

The virus was propagated on MA104 clone 1 cells (ATCC CRL-2378.1) from the kidney epithelium of *Cercopithecus aethiops* (vervet monkey), as follows: cells were infected with the virus suspension and cytopathic effects (CPE) on the cells were observed daily. When the majority of cells were affected (rounded and/or detached) the virus was released by three consecutive freeze-thaw cycles. Cell debris were pelleted by centrifugation at 400 *g* for 10 min, after which the cell-free supernatants were filtered with Amicon-Ultra-15 Ultracel 100K centrifugal filter units (EMD Millipore UFC910024) at 4,000 *g* for 40 min in a swing-bucket rotor centrifuge, to obtain 140-145 µL of concentrated virus suspension. The virus concentrate was then brought to 1 mL

volume in MA104 maintenance media (Table 2.1) supplemented with 5% glycerol (FisherScientific BP229-1). Virus copy number was quantified as number of genomic equivalents by reverse-transcription quantitative PCR (RT-qPCR) using standard-curve-based absolute quantification, aliquoted in volumes of 100 µL containing $4x10^9$ virus genome equivalents (GE), and stored at -80 ºC. To prepare virus suspensions in Peptone-Yeast-Glucose (ATCC 712 PYG) amoebae media, when infected MA104 cells were rounded but not detached, their medium was removed and replaced with desired volume of PYG, in which the virus was harvested directly by consecutive freeze-thaw cycles, as described. Unconcentrated virus suspensions in PYG were aliquoted and stored at -80 ºC for up to two weeks. To assess whether the virus infectivity changed throughout storage in PYG freshly harvested unconcentrated virus in either PYG or in MA104 maintenance medium was used to quantify the infectious virus titer as most probable number of infectious units per mL (MPN IU/mL). In addition, infectious virus titer was quantified for virus stored either at 4ºC or -80ºC for one or two weeks. The efficacy of CVB5 heat inactivation, to obtain deactivated CVB5, was also tested by exposing the virus to temperature ranges of 42ºC – 95ºC for different durations on a heat block (Isotemp Thermo Fisher), after which the presence or absence of infection was observed in triplicates on MA104 cells. Virus inactivation resulting from consecutive freeze-thaw cycles was also evaluated.

2.3 MA104 cell line maintenance and infection

The MA104 cell line was provided by Dr. Xiao-Li Pang. For propagation, the cells were seeded at a density of $7 \times 10^4 – 8 \times 10^4$ cells/mL in custom-made growth media (Table 2.1) in non-vented tissue culture flasks (BioLite 130192 or 130193). When the cells were ~ 80 - 90% confluent they were split by removal of the growth media, rinsed with 1x Phosphate Buffer Saline (PBS; HyClone SH30256.01) and incubated with 1 mL trypsin [HyClone SV30031.01] at 37ºC for 5 min. Remaining un-detached cells were detached from the flask surface using cell scrapers (Falcon 353085) and the trypsin was immediately neutralized by the addition of fresh growth media. Viable cells were counted with Trypan Blue (Gibco 15250-061) dead-cell exclusion on a Hemocytometer (Hausser Scientific Bright-Line 0267151B). The number of cells per mL was calculated as shown in Equation 2.1.

$$\textbf{Number of cells/mL} = (\textbf{average cell count per square}) * (\textbf{dilution factor}) * \textbf{10,000}$$

Equation 2.1 Formula for the calculation of cell count per mL of medium. The average cell count per square refers to all squares, which cells were counted from; the dilution factor was 1:2 in Trypan Blue, while the final multiplication by 10,000 scales up the obtained cell count from the volume of a hemocytometer square to 1 mL of liquid.

MA104 cells were stored in aliquots of 1 x 10^6 – 4 x 10^6 cells/mL in growth media supplemented with 5% dimethyl sulfoxide (DMSO; Sigma D8418) at -80ºC. CVB5 infections of MA104 cells were performed on ~ 80% confluent cells, as follows: for virus propagation, the growth media was removed, and cells were infected with 1 mL of virus suspension (if performed in 25 cm^2 flasks) or 3 mL of virus suspension (if performed in 75 cm^2 flasks). Cells were incubated for 1 h at 37ºC, after which they were rinsed with 1x PBS to remove uninternalized virus and supplied with fresh maintenance medium.

Table 2.1 Formulation of custom-made MA104 media[1]

Growth medium:	**Maintenance medium:**
25 mL of 10x Minimum Essential Medium (MEM; Sigma M0275)	50 mL 10x MEM
25 mL 10x Medium 199 (Sigma M9163)	15 mL sodium bicarbonate
9 mL sodium bicarbonate (Fisher Scientific BP328-1)	5 mL FBS, heat-inactivated
25 mL Fetal Bovine Serum (FBS; Gibco 1780629), heat inactivated	5 mL 100x penicillin-streptomycin (Gibco 15140)
5 mL 100x l-glutamine (Gibco 25030)	425 ml Cell Culture Grade Water
411 ml Cell Culture Grade Water (HyClone SH30529.02).	1 mL HCl
1 mL Hydrochloric Acid (HCl; SA48-1)	

[1] The pH was adjusted aseptically by the addition of 1 mL hydrochloric acid (HCl), which, as advised, adjusts the pH of the described media recipes to optimal (6.5-7) without the need for manual pH measurements and risk of contaminations.

For virus infectious titer quantification, the cells were seeded in 24-well plates (Thermo Scientific 930186) in 1 mL of growth media per well, and incubated at 37ºC, 5% CO_2, and 80% relative humidity (RH). When ~80% confluent, the cells were infected in quadruplicates with serial ten-fold dilutions of experimental virus sample in 500 µL final volumes. Infected cells were incubated for 1 h at 37ºC, rinsed with 1x PBS, supplied with 500 µL fresh maintenance medium, and finally incubated under the same conditions as described for uninfected cells. To avoid uneven evaporation among wells, all infected MA104 well-plates were individually wrapped in cling wrap before being placed in the incubator. All infections, representing replicates of the same experiment, were performed on cells of the same passage number (28-36). In addition, negative cell-culture controls were included, as well as controls from non-virus exposed FLA lysates.

2.4 CVB5 infectivity assays

MA104 cells were infected, as described, in quadruplicates with each one of ten consecutive ten-fold dilutions of experimental virus suspension in MA104 maintenance media. CPE were observed with a light microscope (Olympus) and recorded daily for up to 7 days. At the end of each infectivity assay, the portion of CPE-positive wells for each sample dilution were used for calculation of MPN IU/mL and/or tissue-culture infectious dose 50% ($TCID_{50}$/mL). MPN IU/mL were calculated using a published protocol for the calculation of total most probable number of infectious units, while $TCID_{50}$/mL was calculated using the well-established Reed-Muench formula for the estimation of fifty percent end points. [294,295] Infectious virus titer from FLA co-culture samples was calculated by both MPN and Reed-Muench formulae in parallel. For CVB5 co-cultures with *V. vermiformis* in addition to calculating the infectious virus titer, virus copy number was also quantified by RT-qPCR in order to calculate specific viral infectivity, or the number of virions needed to comprise an infectious unit (Equation 2.2).

$$\frac{(\mathbf{GE/mL})}{(\mathbf{MPN\ IU/mL})} = \mathbf{Number\ of\ virus\ genome\ equivalents\ per\ infectious\ unit}$$

Equation 2.2 Formula for the calculation of the specific infectivity of CVB5

An important clarification about the estimation of specific infectivity is that infectious titer (MPN) estimations do not provide details about the exact number of infectious units but are used as models to estimate the most likely such number. PCR-based assays, on the other hand can detect both infectious and inactivated virions (or even free nucleic acids). The use of the two assays in combination, as described in Equation 2.2, provides the most reliable estimate of changes in virus infectivity over time as it reflects changes in the number of virion counts per infectious unit. It is, however, important to point out that the use of the two assays in parallel is not always plausible, especially for environmental matrices, which may contain PCR inhibitors. Environmental protozoa can provide further challenges for highly sensitive probe-based PCR assays. However, whenever possible, in this research the use of infectious titer estimates was used in combination with quantitative PCR, as described in Equation 2.2, for the most accurate measure of virus infectivity (Section 3.5).

2.5 CVB5 RT-qPCR

All RT-qPCR assays on virus samples were performed on cDNA prepared from freshly extracted RNA, as described. In addition, prior to lysis with the kit-included lysis buffer, pelleted *V. vermiformis* cells were disrupted by continuous pressure through 20-gauge syringe needle (BD 305178). The qPCR reactions were prepared using TaqMan Fast Universal PCR mix (Applied Biosystems 4352042). The total reaction volume of 10 µL contained 1x of the TaqMan master mix, 0.9 µM of selected forward and reverse primer, 0.25 µM of target-specific fluorescent probe, as well as 2.5 µL cDNA template in PCR-grade nuclease-free water (Invitrogen Ambion AM9937). The qPCR primers (EQ for Enterovirus Quantification) and probe used were previously described, [296] and as follows: EQ-1: 5-ACA-TGG-TGT-GAA-GAG-TCT-ATT-GAG-CT-3, EQ-2: 5-CCA-AAG-TAG-TCG-GTT-CCG-C-3, and EQ probe 6-FAM-5-TCC-GGC-CCC-TGA-ATG-CGG-CTA-AT-3-TAMRA. Absolute quantification of number of PCR targets was based on standard curve. The standard curve comprised three serial ten-fold dilutions of 5'UTR dsDNA targets amplified with the EVUTR1 and EVUTR2 primers. Number of dsDNA molecules used for standard curve preparation were quantified by measurement of DNA concentration as described, followed by purification of the PCR product with QIAquick kit (Qiagen 28104), and subsequent calculations following manufacturer instructions for gDNA (genomic DNA) standard curve

preparations. [297] The qPCR standard curve target was named "EVUTR" for Enterovirus Untranslated Region (same as the primers used to prepare it). The assay limit of detection within 95% confidence intervals (LOD_{95}) was calculated by the use of ten replicates of serially diluted EVUTR targets, using a published LOD-calculation spreadsheet, version 4 from 2011, which was originally developed in 2009. [298] The multiple standard replicates had a combined efficiency of 99.8% and an R^2 value = 0.99 (Figure 2.2). The limit of detection was calculated as 33 genomic equivalents (GE) per PCR reaction and therefore the assay was confirmed as highly efficient and sensitive.

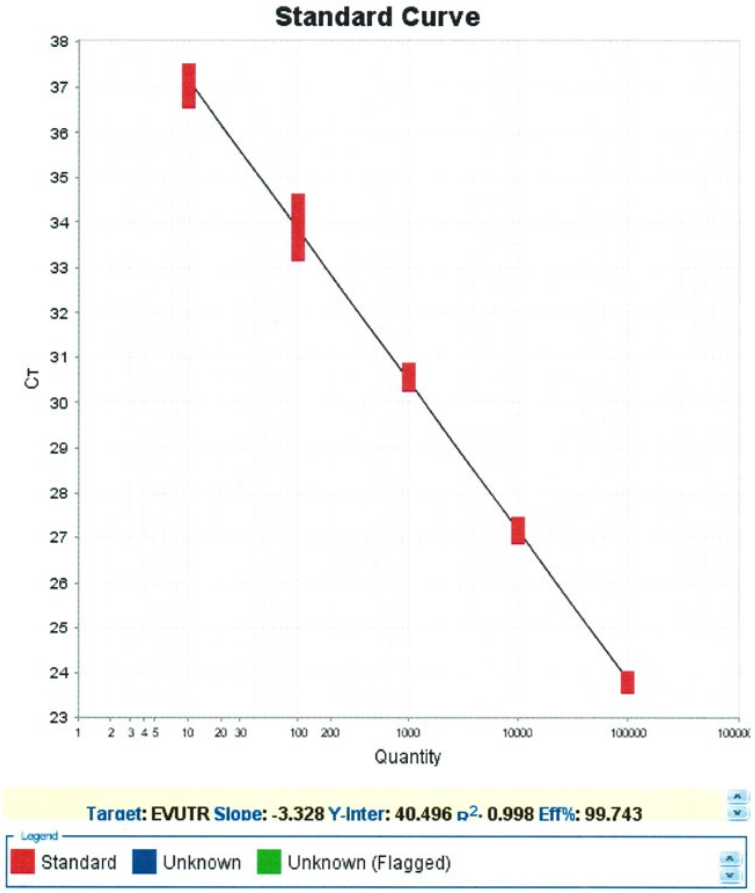


Figure 2.2 Standard efficiency for EVUTR gDNA standards used for qPCR assay validation

Multiple EVUTR dilutions were also used to validate the efficiency of the qPCR assay. Quantification of virus particles as GE/mL was back-calculated based on total GE detected per PCR and respective dilution factors used since RNA extraction. All reactions were run according to manufacturer-recommended fast qPCR protocol, involving an initial denaturation at 95ºC for

20 sec, followed by 45 cycles of two consecutive steps (denaturation at 95ºC for 3 sec, followed by annealing at 60ºC for 30 sec) on an ABI 7500 cycler (Applied Biosystems). All RT and PCR reaction components, except RNA and DNA templates, were handled exclusively in a designated clean room with unidirectional workflow (to avoid nucleic acid contaminations). Nucleic acid templates were handled in a clean biosafety cabinet and prepared in Optical 96-well plates (MicroAmp 4346906) sealed with optical adhesive films (MicroAmp 4311971), or in optical 8-tube strips (MicroAmp 4358293) sealed with optical caps (MicroAmp 4323032). Virus aliquots of 1 x 10^9 GE/mL were stored at -80ºC and used as RNA extraction positive controls for each *V. vermiformis* co-culture experiments where total virus number was quantified, in order to observe the RNA extraction efficiencies among experimental replicates. In addition, some of the same virus aliquots were also used for RNA extraction, and the RNA was then stored at -80ºC for use as RT positive controls, again to compare RT efficiency among experimental replicates.

2.6 Free-living amoebae (FLA)

V. vermiformis (formerly *Hartmannella vermiformis*) Page (ATCC 50237), isolated from a hospital cooling tower, was propagated in custom-made PYG (Peptone-Yeast-Glucose Medium; ATCC 712 medium) or SCGYEM (Serum-Casein-Glucose-Yeast-Extract Medium; ATCC medium 1021), as described (Table 2.2), in non-vented tissue culture flasks. The amoebae were maintained at 25ºC and when they formed fully developed monolayers they were split at a ratio of 1:3 – 1:5 into new flasks with fresh medium for propagation and maintenance. *V. vermiformis* cells were counted as described for MA104 cells and were split with cell scrapers without the use of trypsin. For maintenance and propagation, the amoebae were grown in the serum-rich SCGYEM medium, while in preparation for and during CVB5 co-cultures the amoebae were grown in PYG medium. Aliquoted amoebae (1 x 10^6 cells/mL) were stored in PYG supplemented with 5% DMSO at -80ºC.

A. polyphaga (Puschkarew) Page ATCC 30461, isolated from human corneal scrapings, was propagated in PYG medium in non-vented tissue culture flasks at 25ºC, and similar to *V. vermiformis*, it was split into new flasks when it formed fully grown monolayers to ensure sufficient space for growth that would keep the amoebae actively growing. All experiments on

A. polyphaga were exclusively performed in PYG media. Aliquoted amoebae (1 x 10^6 cells/mL) were stored in PYG supplemented with 5% DMSO at -80ºC. *A. polyphaga* was also tested for growth in various conventional cell-culture media, such as 1x MEM, Eagle's Minimum Essential Medium (EMEM; ATCC 30-2003), Medium 199, and RPMI (Roswell Park Memorial Institute; HyClone SH30027.01) in the presence or absence of serum (FBS).

Table 2.2 Recipes for FLA growth media

ATCC 712 PYG[1]	**ATCC 1021 SCGYEM[2]**
20g Proteose Peptone (BD 211684)	10g Casein (Sigma C6554)
1g Yeast Extract (BD 212750)	2.5g Glucose
0.05M $CaCl_2$ – 8mL (Sigma 223506)	5g Yeast Extract
0.4M $MgSO_4$ – 10 mL (FisherScientific M63-50)	1.325g Na_2HPO_4
0.25M Na_2HPO_4 – 10 mL (FisherScientific BP331-1)	0.8g KH_2PO_4
0.25M KH_2PO_4 – 10 mL (Sigma P5655)	900 mL Distilled Water
1g Sodium Citrate dihydrate (Sigma W302600)	10% FBS (Gibco 1780629)
0.005M $Fe(NH_4)_2(SO_4)_2*6H_2O$ – 10 mL (Sigma 215406)	
900 mL Distilled Water	
2M Glucose – 50 mL (Sigma G8270)	

[1] For PYG medium, filtered glucose was added aseptically after the rest of the ingredients were mixed, autoclaved on a 20-min liquid cycle, and pH adjusted to 6.5 with a Mettler Toledo FEP20 pH meter.

[2] For SCGYEM, the serum-was added to a final concentration of 10% aseptically after the rest of the ingredients were mixed, autoclaved, and filter-sterilized using a Stericup filter unit (Millipore SCGPU05RE).

2.7 Virus-amoebae co-cultures

Virus co-cultures with both *A. polyphaga* and *V. vermiformis* were performed on freshly propagated and actively growing amoebae in serum-deficient PYG medium.

2.7.1 Co-cultures with *V. vermiformis*

V. vermiformis and CVB5 were co-cultured at a ratio of 1:1,000 in 15 mL conical tubes (ThermoScientific 339650). Co-cultures were performed by exposure of 1 x 10^6 amoebae to 25 µL concentrated virus suspension containing a total of 1 x 10^9 virus particles, followed by thorough mixing to facilitate virus-FLA contact. The high ratio was required to ensure sufficient virus available for amoebae feeding for 24 h, after which the co-cultures were washed in PYG media by centrifugation at 4,000 *g* for 5 min to eliminate uninternalized virus. Following the washing step, at days 1, 3, 5, and 7 co-culture samples were collected and centrifuged at 4,000 *g* for 5 min to obtain supernatant and pellet samples. The FLA pellets were additionally washed in PYG media to remove uninternalized virus. Amoebae alone (neg. control), virus alone, and media alone controls were included with each co-culture.

In addition, at each sample collection time point, the growth and encystment rate of *V. vermiformis*, exposed or unexposed to the virus, was quantified by Trypan Blue exclusion as described for MA104 cells. The appearance of *V. vermiformis* monolayers was also observed for FLA co-cultured with infectious CVB5, deactivated CVB5 (95ºC for 10 min) and no-virus control (unexposed amoebae), in order to check for possible cytopathic effects CVB5 could exert on the amoebae. Monolayers were observed with light microscopy in 12-well plates (BioLite 13185), where 15 photos of randomly picked fields of view were taken per well.

Co-culture experiments were performed as independent experimental triplicates, after which the statistical significance of the findings was estimated with two-factor ANOVA analysis, where applicable. To detach the amoebae for sample collection, the tubes were kept on ice for 30-45 sec, after which they were vortexed, and a uniform sample was collected, which was then sub-divided into supernatant fractions and washed pellet fractions. Pellets and supernatant fractions for each time point were stored at -80ºC until the completion of the co-culture experiment, after which the samples were used for virus infectivity assays or total virus quantification with RT-qPCR. To release virus from *V. vermiformis* pellets, the amoebae were disrupted by continuous pressure through a 20-gauge syringe needle.

2.7.2 Co-cultures with *A. polyphaga*

CVB5 co-cultures with *A. polyphaga* were performed as described for *V. vermiformis,* with the following differences:

- Co-cultures were performed in 75 cm^2 non-vented flasks and did not include a washing step after 24 h, in order to avoid stress-response of the amoebae and loss of potentially internalized virus within expelled vesicles.
- *A. polyphaga* were exposed to CVB5 by propagation in virus-containing PYG media, in which the virus was harvested directly from MA104 cells, as previously described.
- Sample collection was performed by disruption of the amoebae monolayers with a cell scraper, after which the co-cultures were mixed thoroughly for the collection of a uniform representative sample. The collected samples were sub-divided into supernatants and pellets, however, the pellet fractions were not additionally washed, as was done for *V. vermiformis*. Samples were collected at day 1, 3, and 5 and stored, together with the no-virus control, at -80ºC for processing after the experiment was completed. To release virus from *A. polyphaga* pellets, the amoebae were disrupted by six consecutive freeze-thaw cycles.
- To test whether *A. polyphaga* could use the virus as a source of nutrient, the amoebae were co-cultured with CVB5 as previously described, with co-cultures performed either in nutritious PYG medium, in nutrient-poor PYG medium (prepared as described here, but without the addition of peptone, yeast extract, or glucose), or in modified Neff's nutrient-poor medium. [170] Neff's nutrient-poor medium was comprised of separately prepared stock solutions in 100 mL distilled water for each of the following ingredients: 1.2 g NaCl (FisherScientific S271), 0.04 g $MgSo_4$, 0.04 g $CaCl_2$, 1.42 g Na_2HPO_4, and 1.36 g KH_2PO_4. The stock solutions were individually autoclaved and combined to form the final nutrient-poor medium. Co-cultures in these media were prepared in 12-well plates, in the presence or absence of CVB5 and were incubated at either room temperature or 37ºC. The appearance of the amoebae was observed daily via light microscopy at the start of the experiment and for up to 2 days, until when starved amoebae have encysted.

2.7.3 Virus presence in FLA after six consecutive washing steps

A. polyphaga and *V. vermiformis* were co-cultured at an MOI of 1,000 in PYG media in 15 mL conical tubes. Samples were collected for RT-qPCR to quantify total virus copy number, immediately at the start of the experiment (t0), as well as after one day (t1). In addition, the virus was quantified from all six wash fractions, and washed amoebae. The experiment was performed in independent triplicates, in order to observe tendencies for virus removal from FLA co-cultures, resulting from extensive washing steps. All collected samples were kept at -80ºC and processed simultaneously for RT-qPCR. Virus was released from the amoebae by subjecting them to 20-gauge syringe pressure, prior to the application of lysis buffer.

2.7.4 Isolation of CVB5 from 20-month old *A. polyphaga* cysts

A. polyphaga co-culture experiments were performed before co-cultures with *V. vermiformis*, and when they were completed, they were stored at 4ºC. This allowed me to perform experiments on them 20 months later, when I could test for CVB5 persistence in mature long-term surviving *A. polyphaga* cysts. To prevent excystment of the amoebae during handling and to ensure that all cysts are mature, the 20-month old cysts were immediately re-suspended in up to 10 mL volume in encystment media (0.1 M KCl (Sigma P9541), 8 mM $MgSO_4$, 0.4 mM $CaCl_2$, 20 mM Tris, 500 mL cell-culture grade water) and maintained overnight at room temperature, after which they were washed in fresh encystment media by centrifugation at 1,000 *g* for 3 min, and re-suspended in 1 mL of fresh encystment media. Washed mature cysts were then treated with 0.5% Sodium Dodecyl Sulfate (SDS; BioRad 161-0301) in 1x PBS, in order to eliminate non-mature cysts and trophozoites. [193] Treated mature cysts were then washed twice more in fresh encystment media to remove cell debris and uninternalized virus, followed by a final wash and re-suspension in 1x PBS. The cysts were disrupted by six consecutive freeze-thaw cycles, and the suspensions were used for infectivity assays to quantify infectious CVB5 titer, with the addition of input virus control, as well as cysts from fresh *A. polyphaga* – CVB co-cultures, which were treated the same way as the 20-month old cysts, in order to investigate virus association with well matured (formed) cysts in both cases (fresh and old co-cultures).

2.8 Fluorescence Microscopy

Virus-amoebae co-cultures were vortexed and transferred onto microscopy cover slips (Fisher Scientific 12-5461) inside 12-well plates, which were incubated at room temperature. After 24 h the growth medium was removed, and the cover-slip-grown co-cultures were then prepared for fluorescence microscopy using an EVOS FL fluorescent cell imaging system (Thermo Fisher Scientific). For *V. vermiformis* samples, fluorescence microscopy was additionally performed at the start of co-cultures (t0).

2.8.1 *V. vermiformis* co-cultures

Cover slip samples were blocked with 1% Bovine Serum Albumin (BSA; Sigma A2058) suspended in modified, serum-free SCGYEM (CGYEM). The samples were blocked for 1 h at room temp, after which they were washed in CGYEM and incubated with mouse-anti-CVB primary antibody (Light Diagnostics 3303) [1:100 in CGYEM] for 45 min at room temp. The samples were then washed twice in CGYEM and incubated with FITC (Fluorescin Isothiocyanate) - conjugated secondary anti-mouse antibody (EMD Millipore AP160F) [1:200 in CGYEM] for 45 min, at room temp. The samples were washed twice more in CGYEM, after which, the amoebae were allowed to recover for 20 min at room temp in SCGYEM (containing heat-inactivated serum). Once the amoebae have recovered, they were fixed in 4% paraformaldehyde (PFA; Sigma 158127) for 3 min at room temp, the fixative was removed, then the samples were mounted with the cover slip directly onto microscopy glass slides with Fluoromount aqueous mounting medium (Sigma F4680) and sealed with transparent nail polish. Images were taken using the EVOS FL system described above. Negative controls (no virus and secondary antibody only), as well as no-fluorescence controls were included to ensure optimal laser exposure settings and no unspecific fluorescence.

2.8.2 *A. polyphaga* co-cultures

The samples prepared on microscopy cover slips, as described, were stained with a modified double-staining protocol (with Rhodamine-conjugated and FITC-conjugated secondary antibodies

sequentially), recommended for the distinction of internalized virus from virus adsorbed to FLA surfaces. [288] The virus-rich PYG medium was removed, the amoebae were washed with 1x PBS, after which they were incubated with anti-CVB primary antibody (Light Diagnostics 3303) for 1 h at room temp and washed again with 1x PBS. The co-cultures were then fixed in 1.5% PFA for 15 min at 4°C. After fixation, the first staining (for internalized virions) was performed again for 1 h at room temp, with Rhodamine-conjugated anti-mouse secondary antibody (Millipore AP124R). The samples were then washed with PBS and stained again with the primary anti-CVB antibody, after which they were washed in 1x PBS and stained with FITC-conjugated secondary anti-mouse antibody (EMD Millipore AP160F). Finally, the samples were washed in 1x PBS, mounted on microscopy glass slides, sealed with nail polish and visualized using EVOS FL system as described, again including a no-fluorescence control. All antibodies used for the double-staining procedure were used at concentrations of [1:100 in 1x PBS]. In addition, despite the use of this double-staining protocol, due to the sensitive nature of *Acanthamoeba* spp. and their rapid encystment, a milder single-staining procedure was also applied, which was as described for *V. vermiformis*, also including a recovery stage.

2.8.3 Primary antibody validation using Western blotting

To confirm the specificity of the primary antibody used for fluorescence imaging of CVB5 in the FLA, the following samples were analyzed by Western blotting: each of the amoebae unexposed to the virus, each one co-cultured with the virus (as described), sterile media, and media with the virus only (10^9 virus GE/mL – same concentration used to initiate FLA-virus co-cultures of an MOI of 1,000 per mL). Input protein alone could not be used as positive control, because the manufacturer of the primary antibody has reported that its epitope is unknown (Light Diagnostics 3303). Co-cultures were performed as described here, and the respective controls (FLA only, virus only, media only) were treated the same way as the co-cultures to avoid variation. After 24 h, the samples were placed on ice for 30 sec to allow for the amoebae to detach from the tubes, but without giving them enough time to encyst. Samples were immediately vortexed thoroughly and an aliquot of 180 µL was mixed with 20 µL of SDS PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) sample loading buffer (1M Dithiotreitol (DTT Fisher BP1725), 2% SDS (BioRad 161-0301), 40% glycerol (Fisher BP229-4), and 0.5% bromophenol blue (Sigma

1081220005) in distilled water). The samples were boiled with the loading buffer at 97ºC on a heat block for 10 min with occasional vortexing every 2-3 min to allow for sufficient protein denaturation. Boiled samples were then allowed to cool down to room temperature, after which 40 µL of thoroughly vortexted aliquots were loaded on a 12% polyacrylamide gel (Table 2.3), together with 2µL of reference dye (Precision Plus Protein Standards – BioRad 161-0373). All samples were loaded with specially-designed gel loading tips (Fisherbrand 02-707-181). Gels were cast on designated 1.5 mm gel casting glassware (BioRad). Resolving gels were cast first and topped with 70% ethanol spray while polymerizing (to burst air bubbles from the surface of the gel and to prevent dehydration), after which the stacking gels were cast on top followed by designated 1.5 mm gel combs (BioRad). For all gels APS (ammonium persulfate BioRad 161-0700) and TEMED (Tetramethylethylenediamine Fisher BP150-20) were used as polymerization catalysts. Gels were run in a BioRad electrophoresis chamber (two gels at a time) in 1x SDS buffer (3 g Tris base, 14.4 g glycine (BioRad 161-0718), and 1 g SDS in 1 L distilled water). SDS PAGE was performed at 25 mAmp per gel and 220 V for 90 min. After electrophoresis, the gel-retained proteins were blotted onto nitrocellulose membranes (BioRad 162-0116) as follows: gels were rinsed gently in distilled water and assembled onto transfer cassettes (BioRad) in the following order: black side of cassette, sponge, filter paper, gel, nitrocellulose membrane, filter paper, sponge, transparent side of cassette. Cassettes were inserted in designated blotting equipment (BioRad) and the transfer was performed for 10 h in Transfer buffer (5.8 g Tris base, 2.9 g glycine, 200 mL methanol (Fisher A452-4), and 800 mL distilled water) at 25 V per gel and 220 mAmp at 4ºC (cold room) with constant slow stirring.

After transfer, the cassettes were disassembled, and gels were separated from the nitrocellulose membranes. The gels were rinsed in gel rinsing buffer (5% acetic acid Fisher A38-212 and 10% ethanol in distilled water) and stained with Coomassie solution (0.3% Coomassie Brilliant Blue (BioRad 161-0400), 50% methanol, 10% acetic acid, 40% distilled water) to check for untransferred proteins. The gels were heated in a microwave for 15 sec, after which they were stained at room temperature until completely blue (approximately 60 min). Stained gels were then placed in rinse buffer, heated for 15 sec, and incubated overnight at 37ºC with constant shaking.
Nitrocellulose membranes were rinsed three times in PBST buffer (0.1% Tween-20 (Sigma P7949) in 1x PBS (Gibco 10010-023) and incubated for 2 h in blocking solution (5% skim milk powder

(Carnation®) in PBST, after which the blocking solution was replaced with primary antibody solution (Light Diagnostics 3303, 1:100 in blocking buffer). Membranes were incubated with the antibody for 60 h at 4°C, after which they were rinsed three times in PBST (5 min each) and incubated with anti-mouse HRP-conjugated (horseradish peroxidase) secondary antibody (1: 5,000 in blocking buffer) for 1 h at room temperature. Membranes were then rinsed three times in PBST and immediately incubated with enhanced chemiluminescence (ECL) substrate solution (BioRad 170-5060) in the absence of light for 5 min. The membranes were then rinsed with PBST once and visualized with GE ImageQuant LAS 4000 imager at developing time of 25 sec (Figure 2.3).

Table 2.3: Recipes for polyacrylamide gels for SDS PAGE

12% resolving gel (15 mL – for two gels)	**4% Stacking gel (5 mL – for two gels)**
1.5M Tris pH 8.8 – 3.75 mL	1.5M Tris pH 8.8 – 625 µL
30% Acrylamide/ Bis-acrylamide solution (BioRad 1001059411) – 6 mL	30% Acrylamide/ Bis-acrylamide solution – 750 µL
10% SDS in distilled water – 150 µL	10% SDS in distilled water – 50 µL
Distilled water – 5.1 mL	Distilled water – 3.575 mL
APS – 50µL	APS – 25µL
TEMED – 50 µL	TEMED – 25 µL

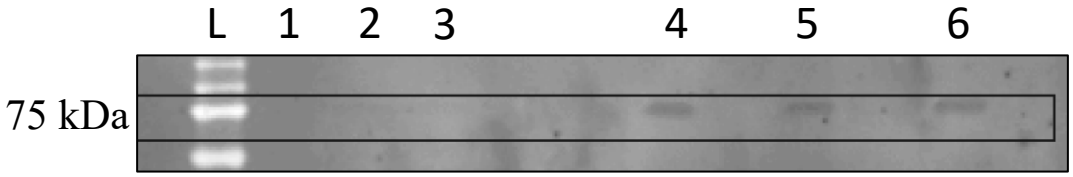


Figure 2.3: Western blot for binding specificity of the Coxsackie B blend reagent. Samples are shown from left to right in the following order: L) Ladder, 1) sterile media, 2) *V. vermiformis* (unexposed to CVB5), 3) *A. polyphaga* (unexposed to CVB5), 4) *V. vermiformis* (co-cultured with CVB5), 5) *A. polyphaga* (co-cultured with CVB5), and 6) virus-only media.

The Coxsackie B blend primary antibody was shown to be specific towards the virus isolate described in this thesis, based on the observation that all virus containing samples (media or

different FLA species) resulted in the presence of bands of the same size and fluorescence intensity, while no other bands were observed on the blot, which serves as confirmation for the antibody specificity especially after 60 h of incubation. The VP1, VP2, VP3, and VP4 of CVB are of molecular sizes of ~ 34 kDa, 30 kDa, 26 kDa, and 8 kDa respectively, therefore the detected target (Figure 2.3) appears to be a dimer or a multimer, however, because the manufacturer has not disclosed the antibody epitope it is difficult to conclude which virion protein/s are being detected. [299]

2.9 Transmission electron microscopy (TEM)

Co-cultures of CVB5 and *V. vermiformis* were initiated as described and grown on Thermonax cover slips (Thermo Fisher 174985). Non-CVB5 exposed amoebae were also included as negative controls. After 24 h, the samples were fixed with an electron microscopy fixative (Electron Microscopy Sciences 15960) and submitted for processing at the imaging core at the Faculty of Medicine and Dentistry, University of Alberta, where the samples were stained with osmium tetroxide and uranyl acetate, contrasted, sectioned into 70 nm sections and carbon coated with 4.47 nm carbon fibers. Images were obtained with a Hitachi H-7650 transmission electron microscope, whose software measuring tool was used to measure the size of virus-like particles observed inside *V. vermiformis*.

Chapter 3: Results

3.1 Identity of the clinical *Enterovirus* B isolate

The clinical *Enterovirus* B isolate was obtained from patient stool samples by trained clinical lab personnel and details about its isolation were purposefully omitted to comply with Alberta Health Services (AHS) regulations. For this research the isolate was obtained from cell culture supernatants and its identity was confirmed via sequencing of the 5' untranslated region (UTR). The 510 bp portion of the 5'UTR is shown below, together with the contig (highlighted), which were analyzed with NCBI online Megablast tool to find similarities to published genome database.

5'-
TGTTTTAAAACCCTCTCCCCAATTTGAAACTTAGAAGCAATACACCTCGATCAATAG
TAGGCATGACACGCCAGCCATGTCTTGAT**CAAGCACTTCTGTTTCCCCGGACTGA**
GTATCAATAAACTGCTTGCGCGGTCGAAGGAGAAAACGTCCGTTACCCGACTA
ACTACTTCGAGAAACCCAGTAACACCATGGAAATTGCGGAGTGTTTCACTCAGC
ACATTCCCAGTGTAGATCAGGTCGATGAGTCACCGCATTCCCCACGGGTGACC
GTGGCGGTGGCTGCGCTGGCGGCCTGCCCATGGGGCAACCCATGGGACGCTT
CAATATGGACATGGTGTGAAGAGTCTATTGAGCTAGTTAGTAGTCCTCCGGCC
CCTGAATGCGGCTAATCCTAACTGCGGAGCACGTGCCTCCATTCCAGGGGGTG
GCGTGTCGTAACGGGCAACTCTGCAGCGGAACCGACTACTTTGGGTGTCCGTGT
TTCTTTTAATTTTATACTGGCTGCTT -3'

The contig sequence was identified as coxsackievirus B5 (CVB5) Faulkner strain, with a sequencing score of 708 out of 708 total, 100% sequence identity, and an E-value of 0. The high specificity of the Megablast result was only observed for the Faulkner strain of CVB5. When the full 510 bp sequence was analyzed, the same result was obtained, with 100% identity, an E-value of 0, and a sequence score of 942 out of 942 total, which therefore confirmed the strain as a CVB5 isolate.

3.2 Virus infectivity in different storage media and temperatures

Because *A. polyphaga* co-cultures were performed in virus-containing PYG media, where virus stocks were aliquoted and stored for use between co-culture replicates, changes in virus infectivity resulting from virus storage in PYG over time, were compared to storage in the MA104 maintenance medium (Figure 3.1).

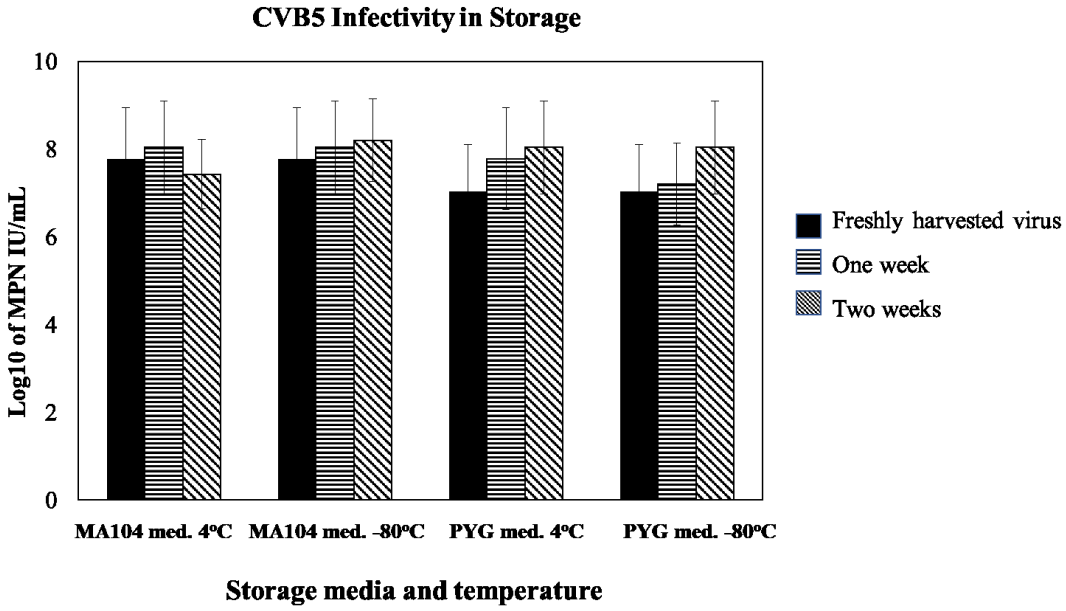


Figure 3.1 CVB5 infectivity in PYG and MA104 cell media. Results are plotted as the most probable number of infectious units (MPN IU) per mL in either media (PYG optimal for amoebae or MA104 cell line maintenance medium) from 4ºC or -80ºC storage temperatures. The error bars represent 95% confidence intervals from experimental duplicates (n = 2). Based on a two-factor ANOVA, $P = 0.039$ among sample replicates, and $P = 0.428$ among storage conditions.

In addition, because heat is one of the obstacles for persistence in treated water distribution systems, such as hot water tanks, the heat resistance of the clinical isolate was evaluated by testing its deactivation at the recommended temperatures of 42ºC (according to the Coxsackievirus Pathogen Safety Data Sheet) [300] or higher, including protein denaturation conditions (95ºC for 10 min) to test how likely the isolate would be to persist in high-temperature environments, such as

hot water tanks (Table 3.1). Heat resistance is important in the context of outbreaks and epidemiological significance of the isolate. Loss of virus infectivity resulting from multiple freeze-thaw cycles was also evaluated. Presence or Absence of CPE caused by treated CVB5 were observed on MA104 cells in triplicates (Table 3.1). Of concern, heat treatment of CVB5 at recommended inactivation temperatures and higher (72-95ºC for 10 min) did not result in loss of infectivity. Successful deactivation was only possible at protein denaturation conditions, and therefore only through physical disruption of the virus capsid. Numerous freeze-thaw cycles also did not inactivate the CVB5 isolate.

Table 3.1 CVB5 resistance to heat inactivation and freeze-thaw cycles. The presence (+) or absence (-) of cytopathic effects was observed on MA104 cells in triplicates

Inactivation Method	**CVB5 Infectivity on MA104 cells**	**Inactivation Method**	**CVB5 Infectivity on MA104 cells**
42ºC for 5 min	+ + +	72ºC for 5 min	+ + -
42ºC for 10 min	+ + +	72ºC for 10 min	+ + +
52ºC for 5 min	+ + +	**95ºC for 10 min (protein denaturation)**	**- - -**
52ºC for 10 min	+ + +	3 freeze-thaw cycles	+ + +
62ºC for 5 min	+ + +	6 freeze-thaw cycles	+ + +
62ºC for 10 min	+ + -		

3.3 FLA growth in different liquid media

Previous studies on virus-amoeba interactions have reported contradictory findings due to different methods used, but also the fact that they performed liquid cultures in cell-culture media is concerning. [289–291] Liquid co-culture experiments need to be performed using actively feeding amoebae (via pinocytosis), which could be challenging in cell culture media, as compared to more optimal FLA growth media. Many commonly used cell culture media did not provide sufficient nutrition to environmental FLA tested here, unless supplemented with serum (Table 3.2). Most importantly, the RPMI medium, which was used for some of the contradictory findings on virus internalization by *A. polyphaga*, did not support normal growth of the test amoebae. Interestingly, Medium 199 supplemented with FBS supported optimal growth of the amoebae, like no other cell-

culture medium tested, however Medium 199 is a growth supplement, which can support the establishment of some primary, non-immortalized cell cultures. These observations emphasized the difference between amoebae and established laboratory cell lines, and the importance of using optimal amoebae growth media.

Table 3.2 Growth of *A. polyphaga* in optimal media compared to classical cell-culture media

Liquid medium	***A. polyphaga* growth**	**Liquid medium**	***A. polyphaga* growth**
MA104 maintenance media	No growth	EMEM 1x + 1% FBS	No growth
MA104 growth media	No growth	EMEM 1x + 5% FBS	No growth
MEM 1x	No growth	Medium 199	Limited growth
MEM 1x + 1% FBS	No growth	Medium 199 + 1% FBS	Limited growth
MEM 1x + 5% FBS	Limited growth	**Medium 199 + 5% FBS**	**Optimal growth**
RPMI	Limited growth	**PYG**	**Optimal growth**
EMEM	No growth		

3.4 CVB5 persistence in FLA after extensive washing

Washing steps are important in order to remove uninternalized virus for more accurate assessment of FLA-virus association. Washing steps were included in existing reports of contradictory findings, [289-291] however extensive washing can remove expelled food-containing amoebae vesicles, therefore excessive washing could interfere with the accuracy of virus detection from FLA co-cultures as result of potential loss of virus resulting from FLA washing. To address this, CVB5 was co-cultured with both *A. polyphaga* and *V. vermiformis* subject to extensive washing steps, and inconsistencies were observed in virus detection from different wash fractions for the same amoebae (Figure 3.2). The experiment, however, also showed that virus genome equivalents could still be detected in thoroughly washed amoebae (six washing steps) thus indicating virus internalization or tight adsorption to both *A. polyphaga* and *V. vermiformis* (Figure 3.2). The RT-qPCR standard efficiency was 97.6% with an R^2 value of 0.999, while the respective RT and PCR negative controls were either negative or below the limit of detection, and therefore the qPCR assay was efficient and sensitive throughout the washing-steps experiment. On the other hand, these results show, that even after extensive washing, CVB5 genomes were detected in association

with *A. polyphaga* and *V. vermiformis* pellets (Figure 3.2), especially in *A. polyphaga,* where virus was detected from washed pellets in all three experiments, therefore confirming internalization of the virions, or strong adherence to the FLA surface.

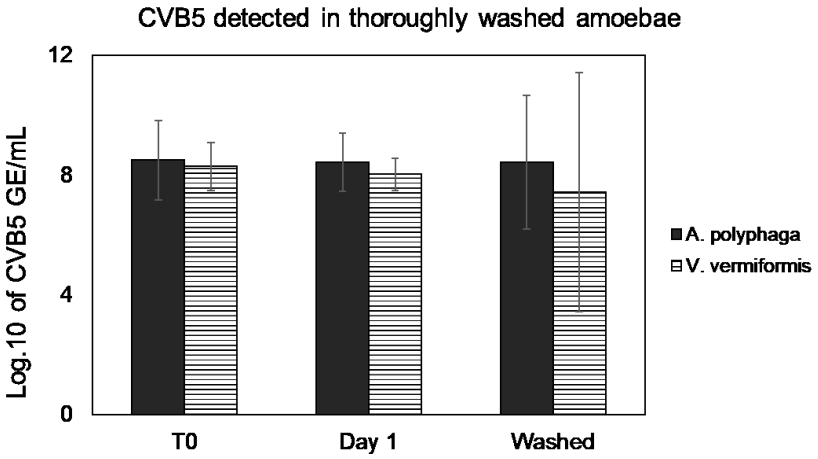


Figure 3.2 CVB5 persistence in *A. polyphaga* and *V. vermiformis* throughout extensive washing steps. Virus genome equivalents were quantified immediately after the start of co-cultures (t0), one day later before washing (t1), as well as at the end of the six consecutive washing steps. RT and qPCR negative controls were negative or below the limit of detection. (n = 3 for washing experiments on each amoeba, where each data point replicate was subject to a single RT-qPCR assays). Based on a single-factor ANOVA, $P = 0.367$ for virus detected in *A. polyphaga*, while $P = 0.086$ for CVB5 detected from *V. vermiformis.*

3.5 CVB5 persistence in FLA over time

3.5.1 Virus persistence as quantified genome equivalents

To determine how much virus is detected over time in co-culture with each amoeba, as compared to input virus (*i.e.* virus persistence over time), absolute quantification of virus genome copy number was possible due to the sensitive RT-qPCR assay, however environmental amoebae could provide interference with probe-based assays similar to other environmental samples, due to the presence of FLA metabolites, which could be PCR inhibitors. [160,213,216,301] Optimized culture conditions allowed for reproducible RT-qPCR data on *V. vermiformis* co-cultures with CVB5,

however samples from *A. polyphaga* co-cultures were more challenging and highly variable among experimental replicates, even when RT-qPCR was performed with different primer-probe sets (the one described here, as well as one obtained from a ProvLab protocol). As a result, contrary to the previously described wash-step experiments, where virus was detected in both amoebae, absolute quantification of virus particles over time in co-culture was only achieved with *V. vermiformis* (Figure 3.3a). The majority of virus genomes were detected in co-culture supernatants and, as compared to the input virus, the virions associated with washed FLA pellets were almost three $\log_{10}$ less in number. Virion quantity associated with washed *V. vermiformis* pellets, however, could successfully cause infection to susceptible hosts (Figure 3.3a). The observation was consistent among independent co-culture replicates with comparable RNA extraction and reverse transcription efficiencies (Figure 3.3b) as well as comparable standard curve efficiencies (Figure 3.4).

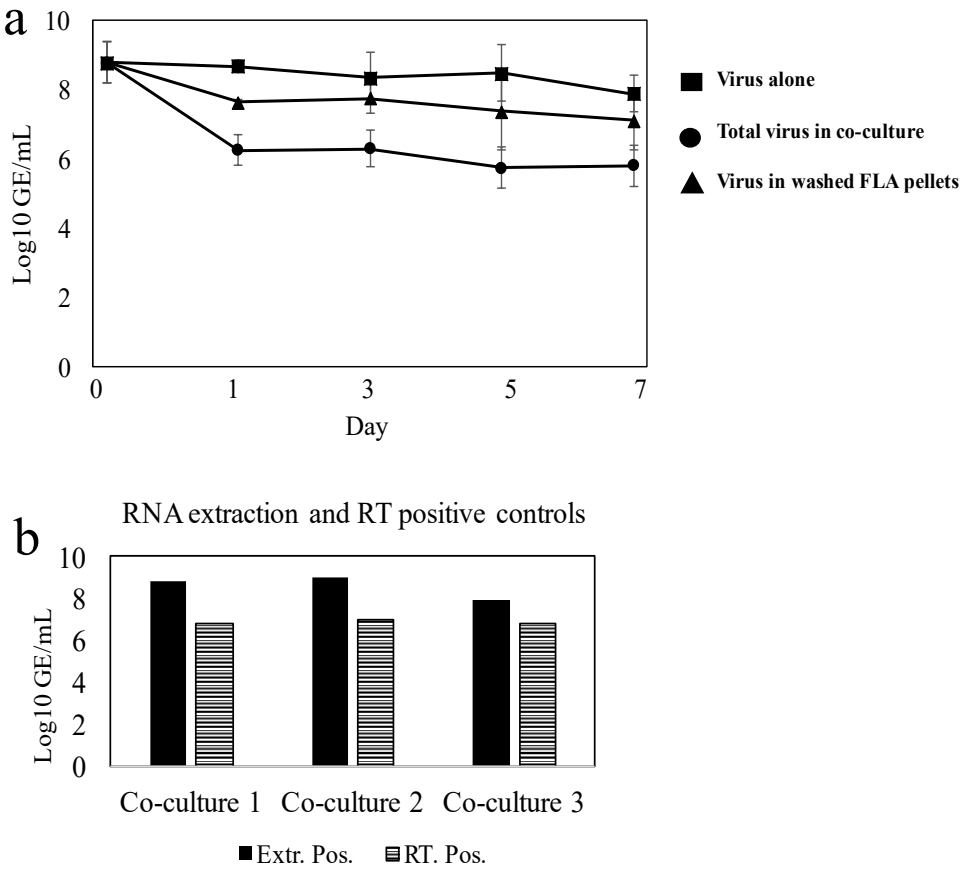


Figure 3.3 CVB5 copy number quantified with RT-qPCR. In (a) data were obtained from independent experimental triplicates (n = 3 for each displayed data point, where each data point

replicate was subject to a single RT-qPCR assay) and plotted as genomic equivalents per mL (GE/mL). Error bars represent one standard deviation. Based on a two-factor ANOVA among days and among sample types (virus alone, total virus, pellet) $P < 0.05$. Co-culture negative controls (FLA unexposed to the virus) and RT-qPCR controls (nuclease-free water used for RT and qPCR master mix preparation) were negative or below the limit of detection (33 qPCR targets). In addition, RNA extraction and RT positive controls were used to evaluate the RNA extraction and RT efficiencies among the experimental replicates (b). Because only a fraction of extracted RNA was used for RT, the RNA extraction controls and RT controls appear on different scales and were not corrected for dilution factor in order to separately visualize differences.

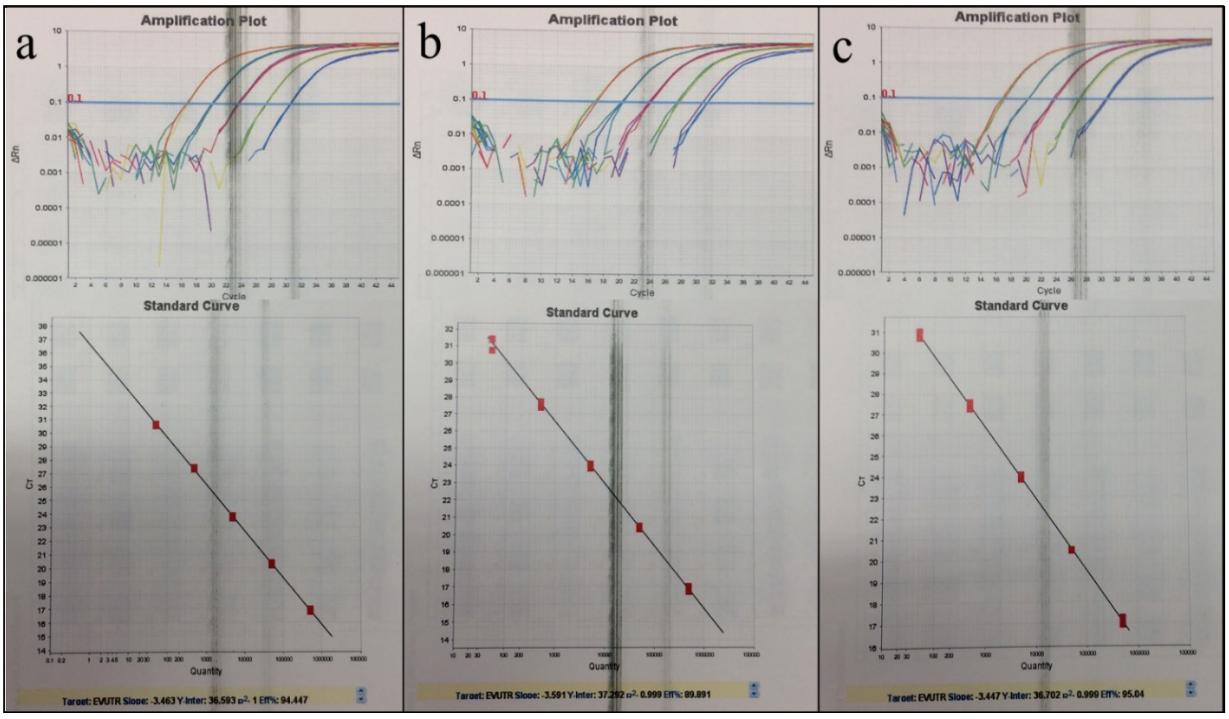


Figure 3.4 RT-qPCR standard curves used for virus quantification in the three experimental replicates of co-cultures with *V. vermiformis*. Standard efficiencies were 94.5% (a), 99.9% (b), and 95% (c), with R^2 values of 1, 0.999, and 0.999 respectively. Standard curves for each experimental replicate were performed in triplicates (n=3 for each displayed standard curve data point).

3.5.2 Virus persistence as infectious titer

Despite PCR challenges with *A. polyphaga* co-cultures, CVB5 persistence in both amoebae could still be quantified using cell culture infectivity assays (Figure 3.5) due to the fact that amoebae lysates did not exert any visible cytopathic effects on MA104 cells in the absence of virus, when diluted in cell culture media. The infectious virus titer associated with pellet and supernatant fractions of both *A. polyphaga* and *V. vermiformis* was plotted as total most probable number of infectious units (MPN IU/mL), or as 50% tissue-culture infectious dose ($TCID_{50}$/mL) to compare what fraction of the total infectious virus units present in a sample is required to infect 50% of cultured cells (Figure 3.5). This provided insights about the infectious potential of the virus even in the absence of RT-qPCR data for *A. polyphaga* co-cultures.

The number of infectious CVB5 units associated with both *V. vermiformis* and *A. polyphaga* remained consistent throughout the duration of the experiments, and in both cases the infectious virus titer from pellets was significantly lower as compared to total co-cultured CVB5 (Figure 3.5). The input virus at the beginning of the assay had a higher infectious potential in *A. polyphaga* co-cultures, as observed from the difference in MPN vs. $TCID_{50}$ data, which could be due to virus harvesting in PYG straight from MA104 cells for co-cultures with *A. polyphaga*. When MPN and $TCID_{50}$ values are the same all of the available number of infectious units were required to infect 50% of the cells, but when the $TCID_{50}$ value was lower than the total number of MPN IU, the virus had a higher infectious potential. Nonetheless, regardless of this apparent difference associated with each method, starting at day 1, the infectious potential for CVB5 in co-culture with *A. polyphaga* was very similar. These results suggested that co-cultured virions, especially ones associated with pellets, suffered a similar impact on their infectious potential, yet infectious virions did persist over time in co-culture with both amoebae. An interesting observation for both amoebae, was also that the total infectious CVB5 titer at day 5 of co-cultures was almost identical. Five days without fresh medium supply is normally when most FLA species would begin encysting due to the depletion of nutrients or space for growth, therefore this could be a limitation of the liquid co-culture assays, where virions are released and suspended in the liquid medium, which was therefore left un-exchanged throughout the duration of the experiments.

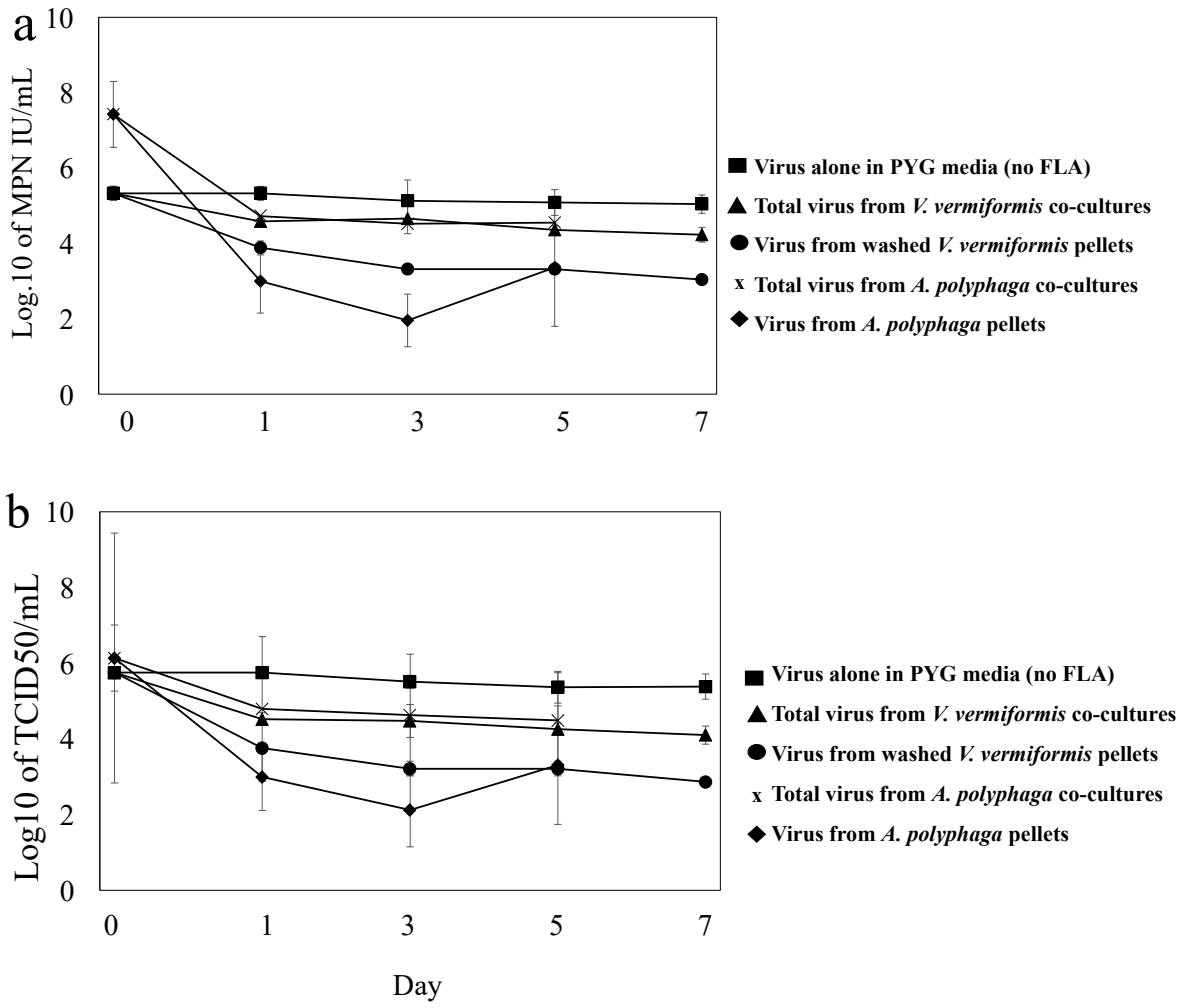


Figure 3.5 CVB5 infectivity over time in co-culture with *A. polyphaga* and *V. vermiformis*. Infectious virion titers estimated as (a) Log_{10} most probable number (MPN) infectious units per mL (MPN IU/mL) or (b) Log_{10} tissue culture infectious dose 50% (TCID50). Data were obtained from independent experimental triplicates for *V. vermiformis* (n = 3 for each displayed data point) and six replicates for *A. polyphaga* (n = 6). Each data point was obtained from quadruplicate serial dilutions. Error bars represent one standard deviation. All negative culture controls were negative for cytopathic effects. Based on a two-factor ANOVA in a) for *A. polyphaga* among sample types (virus alone, pellet, supernatant) $P < 0.05$ and among time points $P = 0.512$; for *V. vermiformis* among sample types (virus alone, pellet, supernatant) $P < 0.05$ and among time points $P < 0.05$. In b) for *A. polyphaga* among sample types $P = 0.052$; and among time points $P = 0.802$; for *V. vermiformis* among sample types $P < 0.05$ and among time points $P < 0.05$.

3.5.3 Changes in viral specific infectivity over time with *V. vermiformis*

The combination of RT-qPCR and linear regressions (for MPN IU calculation) allowed further evaluation of changes in virus infectivity resulting from internalization by *V. vermiformis*. The two assays allowed estimation of the number of virions needed to comprise an infectious unit (as described in Equation 2.2) and how this number changed over time, in the presence or absence of *V. vermiformis*. In other words, this is a more reliable way of determining whether it takes a larger or smaller number of virions to result in the same infectious outcome. The specific infectivity of CVB5 co-cultured with the amoebae, and especially associated with pellets, was about 0.5 – 1 $\log_{10}$ less as compared to the number observed for the input virus and as compared to total virus in co-culture (Figure 3.6), which suggested, together with the infectivity data (MPN and $TCID_{50}$), that internalization of CVB5 by *V. vermiformis* can impact the infectivity of CVB5, however, despite that infectious virions were detected throughout the entire duration of the 7-day co-culture experiments.

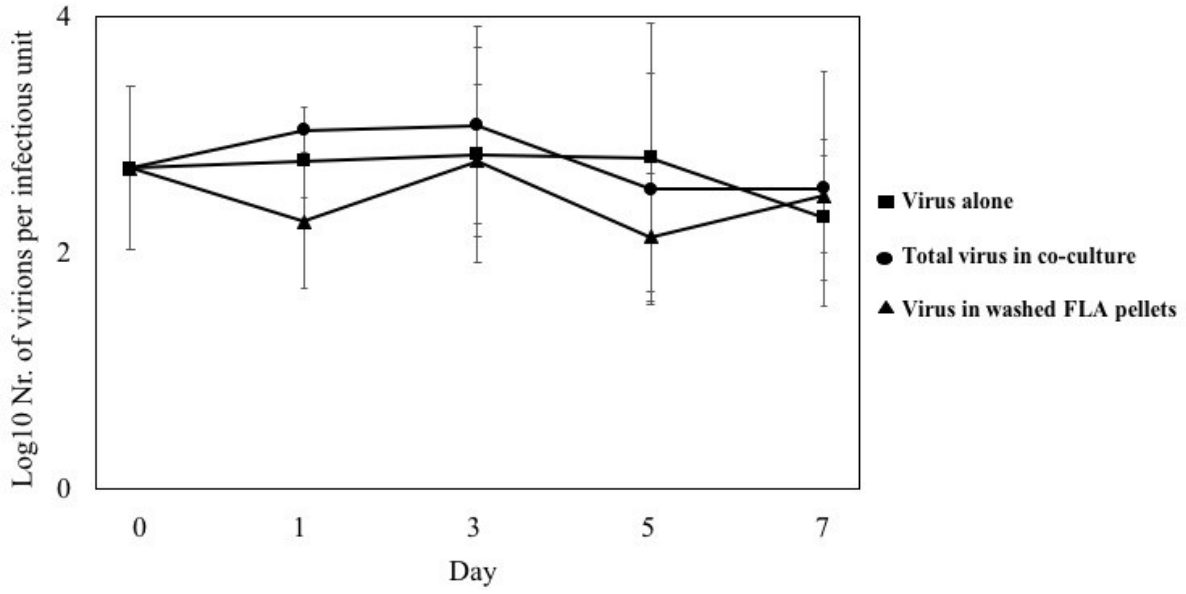


Figure 3.6 Specific infectivity of CVB5 in co-culture with *V. vermiformis*. The data are obtained from the three independent *V. vermiformis* co-culture replicates show in Figures 3.3 and 3.5, using the formula described in Equation 2.2. Error bars represent one standard deviation. Based on a two-factor ANOVA, among sample types (virus alone, supernatant, pellet) $P = 0.501$ and among time points $P = 0.677$.

3.5.4 Assessment of possible cytopathic effects of CVB5 on the amoebae

The appearance of *V. vermiformis* monolayers over time in co-culture with CVB5, with deactivated CVB5, or with no virus, did not differ visibly (Figure 3.7a). In addition, the presence of virus did not seem to affect the growth and encystment of *V. vermiformis* over time (Figure 3.7b).

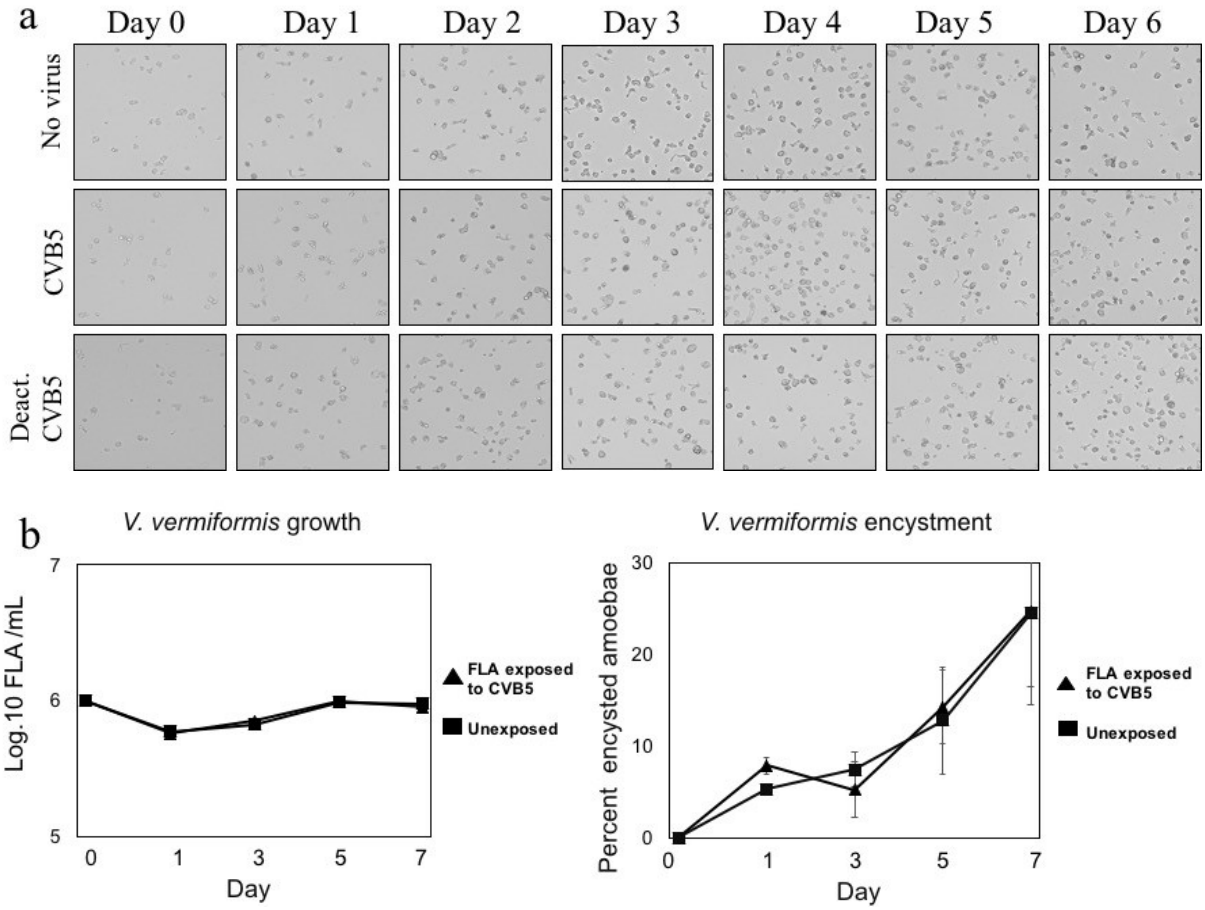


Figure 3.7 Effects of CVB5 on *V. vermiformis*. In (a) *V. vermiformis* cultured with either CVB5, deactivated CVB5, or no virus over time; 15 random fields of view photos were taken per well. In (b) Growth and encystment rate of *V. vermiformis* exposed to CVB5 or unexposed to the virus. Each data point represents independent experimental triplicates (n = 3). Based on a two-factor ANOVA for FLA growth with or without CVB5 present $P = 0.78$, while for growth measured over time $P < 0.05$; For encystment rates in the present or absence of CVB5 $P = 0.826$ and over different time points $P < 0.05$.

Similar to what was observed for *V. vermiformis, A. polyphaga* monolayer appearance did not differ visibly for amoebae cultured with infectious CVB5, deactivated virus, or no virus (Figure 3.8a). In addition, the virus did not seem to affect the growth and encystment of *A. polyphaga* either (Figure 3.8b). Therefore, CVB5 did not cause any apparent cell injury in either of the two species of amoebae tested.

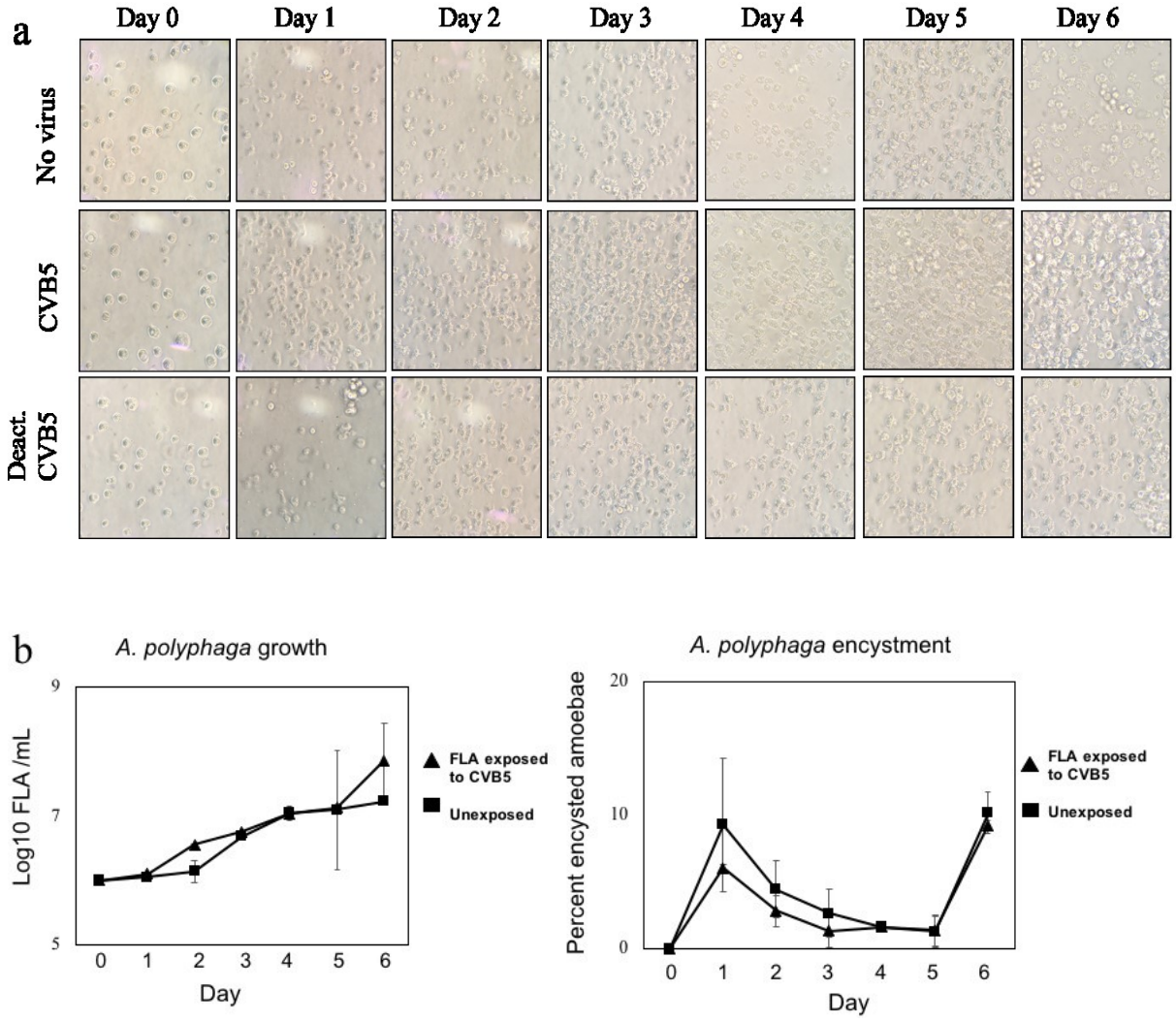


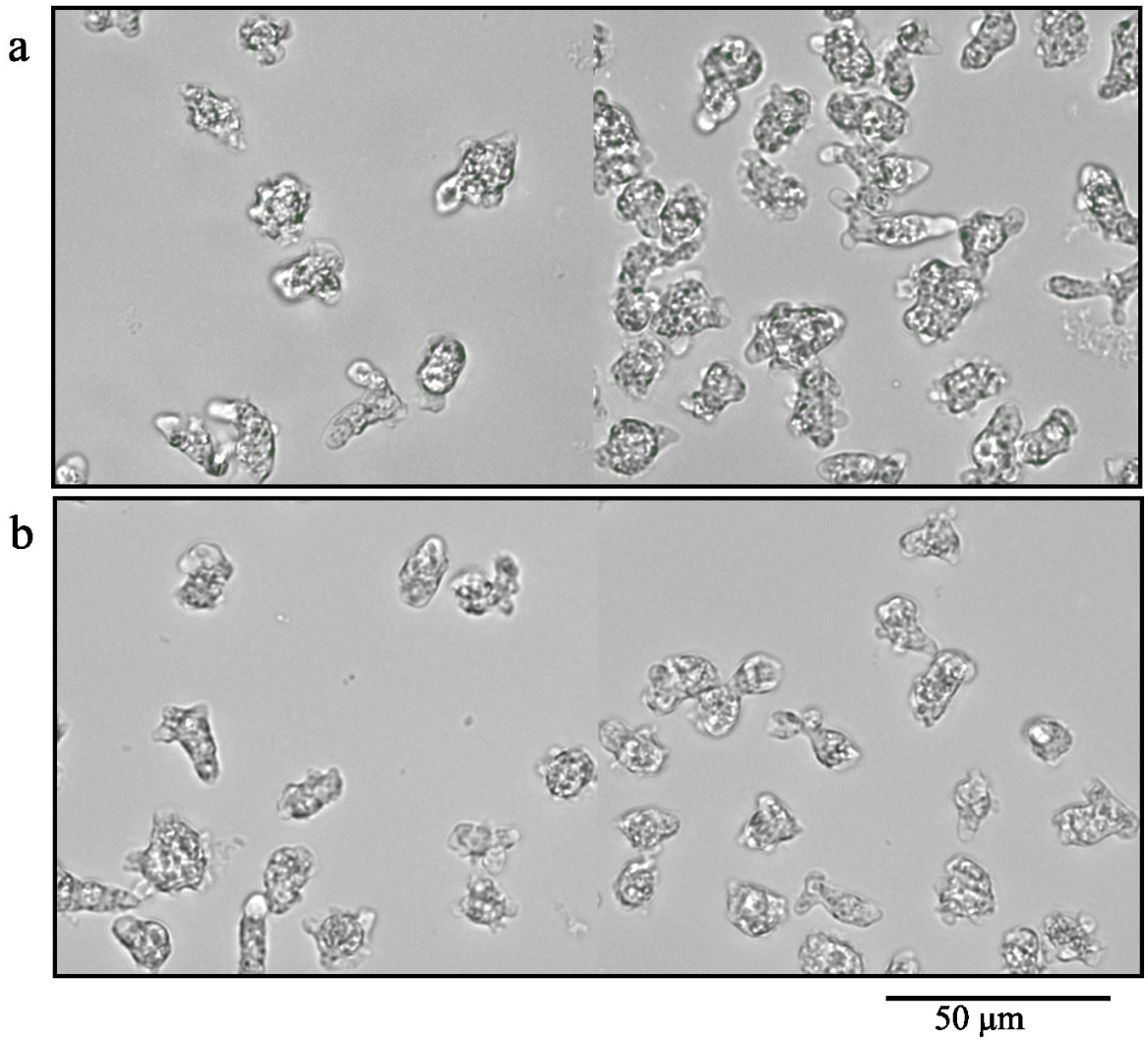
Figure 3.8 Effects of CVB5 on *A. polyphaga*. *A. polyphaga* appearance (a), as well as growth and encystment over time (b) in the presence or absence of CVB5 show no visible cytopathic effects from the virus. In (a) 15 random photos were taken per field of view per well. In (b) data were obtained from independent experimental triplicates (n = 3) and error bars represent one standard deviation. Based on a two-factor ANOVA for FLA growth with or without CVB5 present $P <$

0.05, while for growth measured over time $P = 0.017$; For encystment rates in the present or absence of CVB5 $P < 0.05$ and over different time points $P = 0.018$.

3.5.5 Virus localization in *V. vermiformis*

Virus was detected by immunofluorescence microscopy from co-cultured *V. vermiformis* (Figure 3.9). Because of the small size of *V. vermiformis*, however, a higher magnification was required to visualize the virus-associated fluorescence signal, an observation made for all life stages of the amoeba, including tightly associated with cysts (adsorbed or internalized), individual trophozoites or trophozoites in monolayers, as well as expelled vesicles (Figure 3.10). Fluorescent signal was detected at the start of co-cultures, at day 1 and up to day 4, however the signal was weaker at day 4. For better visualization of CVB5 in *V. vermiformis*, TEM imaging was also performed, where amoebae exposed to and unexposed to CVB5 were compared (Figure 3.11 – Figure 3.15). Virus-like particles were observed within intact *V. vermiformis* with well-shaped overall structure, including pseudopods and mitochondria (Figure 3.11a and b). A higher magnification of the particles revealed that they have icosahedral-like shapes and tend to form small aggregates with one another (Figure 3.11c). Their size was comparable to the expected CVB5 virion size, however, the particle sizes were also measured using the Hitachi TEM software measuring tool (Figure 3.12 and Figure 3.15) and therefore further confirming that the size of the particles was around the expected ~ 30nm for coxsackieviruses. Virus particles were also observed in replicating *V. vermiformis* cells (Figure 3.13) and therefore due to the ability of co-cultured amoebae to form pseudopods and replicate, no apparent cell injury was observed as result of internalization of CVB5 (Figure 3.11, Figure 3.13, and Figure 3.14). Furthermore, what was observed here for the first time with *V. vermiformis* was that internalized CVB5 particles could be contained inside and/or expelled within vesicles in the surrounding of the amoeba (Figure 3.14) - an observation made for independent TEM imaging sessions from independent co-cultures. To take it one step further, virion-like (similar shape and/or size) particles were compared among co-cultured vs non-CVB5 exposed *V. vermiformis* (Figure 3.15). Due to the small size of picornaviruses, like coxsackievirus, and due to the presence of ribosomes in actively feeding amoebae (which are most likely to actively internalize virions from their growth media), it is important to distinguish virus-like particles from other cytoplasmic content, such as ribosomes, granular cytoplasm, etc. Therefore, the measuring

tool on the Hitachi TEM software was used to measure the size of particles, which were suspected to be virus-like in appearance, in both unexposed to CVB5 as well as amoebae co-cultured with CVB5 (Figure 3.15). Particles of ~ 30nm diameter were only observed in co-cultured *V. vermiformis* (Figure 3.15b, d, f), while particles observed for non-CVB5 exposed amoebae were ~ 17nm in diameter (Figure 3.15a, c, e), and therefore these were most likely ribosomes, or other amoeba-specific cytoplasmic structures. In combination with the observation that the CVB5-like particles had an icosahedral-like shape with differentially contrasted equally distributed spots (probably capsid depressions), as well as a tendency to form small non-linear aggregates (Figure 3.11c), the data demonstrated that the observed particles were virions internalized by *V. vermiformis* (and contained and expelled within the digestive or other vesicles of the amoeba).



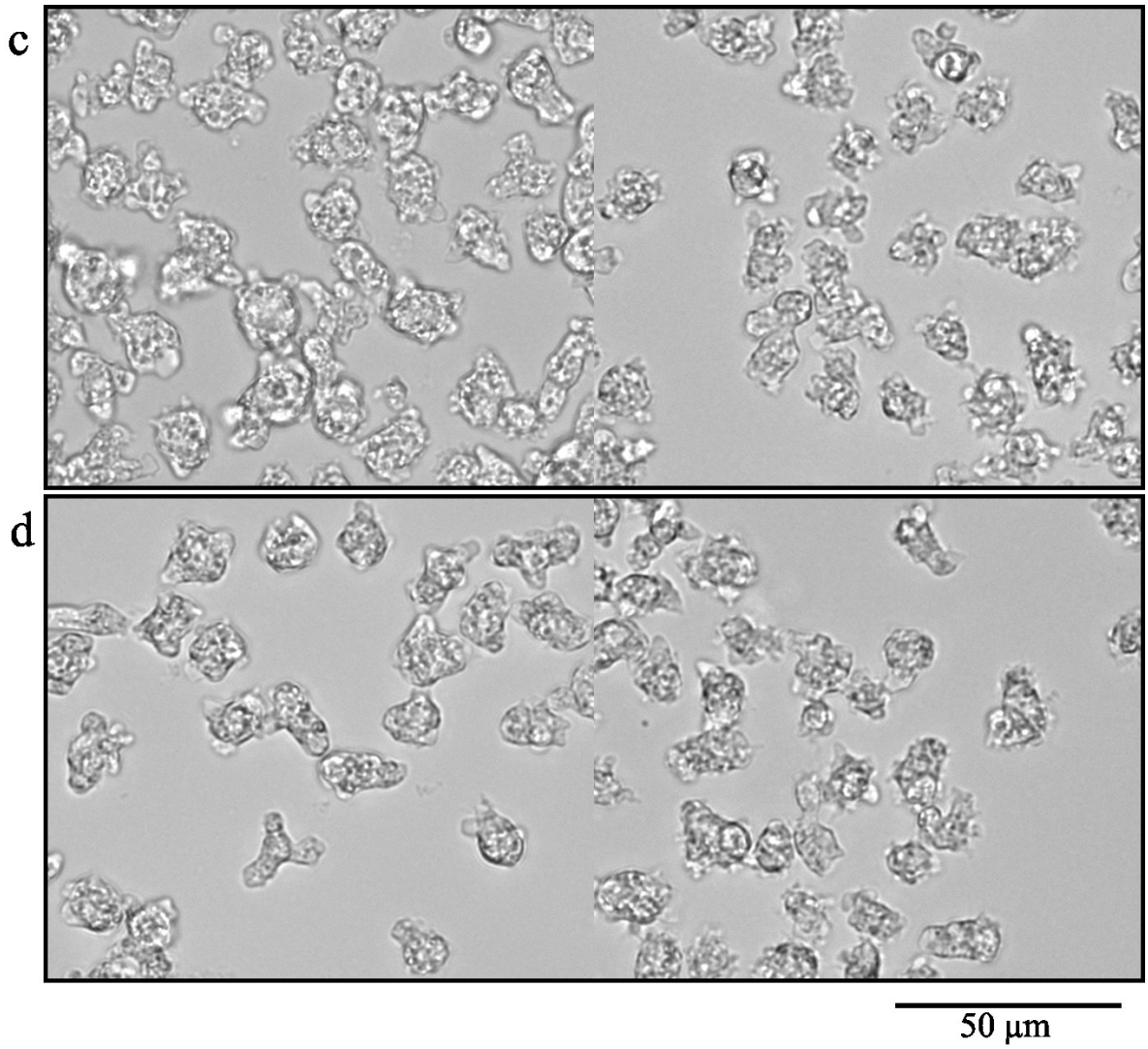


Figure 3.9: Fluorescence imaging of CVB5 in co-culture with *V. vermiformis.* MOI = 1,000; 1 dpi. Two photos shown in each section (a – d) out of 5 random fields per view. In a) CVB5 was stained and visualized as described here and compared to negative controls (non-exposed amoebae) in b). Both co-cultured and non-CVB5 exposed amoebae, were also each stained with secondary antibody only (c and d respectively) to check for unspecific fluorescence.

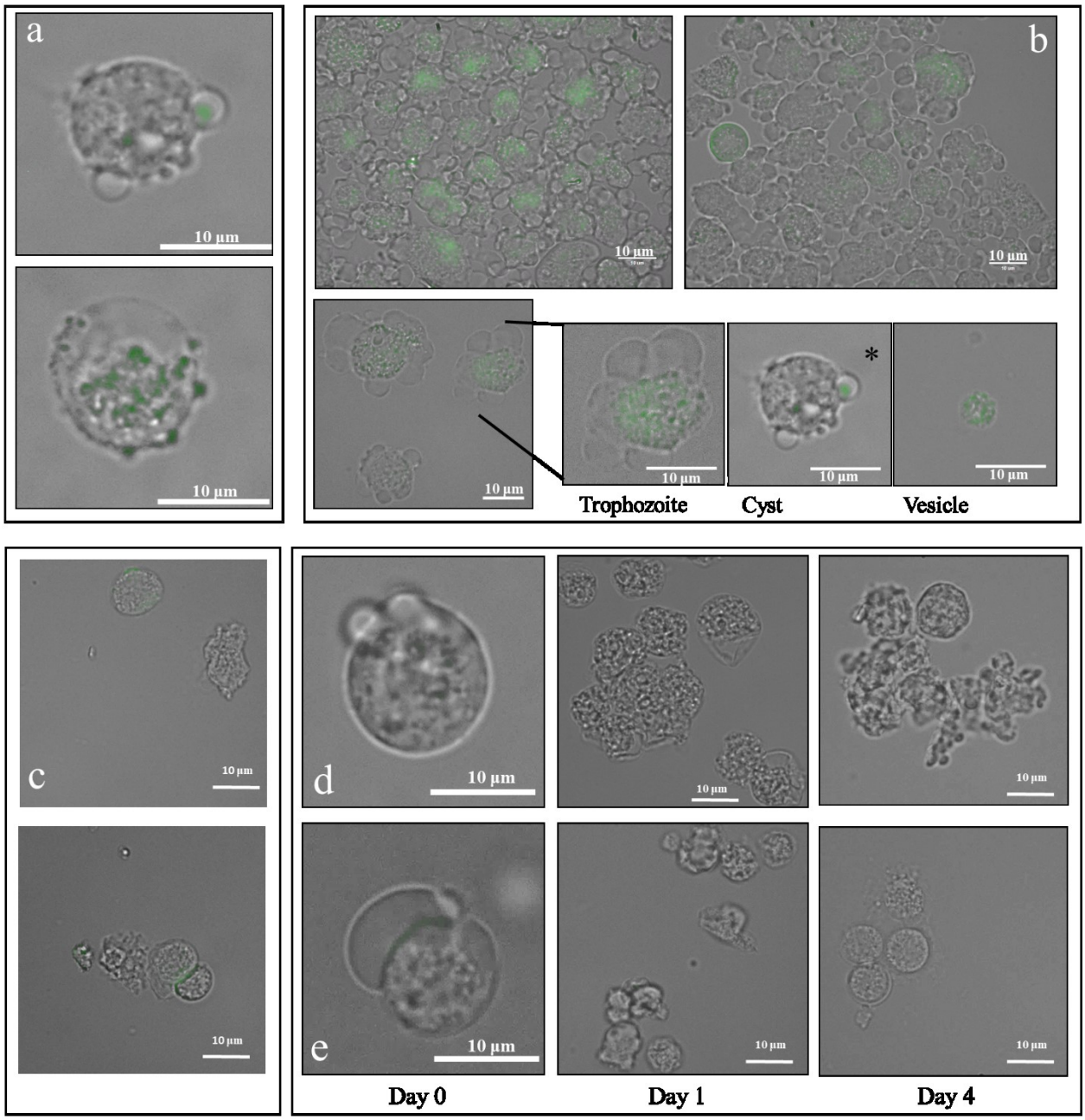


Figure 3.10 High-magnification fluorescence imaging of *V. vermiformis* and CVB5. The virus was immuno-labeled with FITC-conjugated IgG-s at (a) day zero, (b) day one and (c) day 4 of co-culture; size ~ 30 nm diameter. * The image of the cyst from day zero is included for size comparison next to the trophozoite and the expelled vesicle observed at day one (b). No unspecific fluorescence was observed in the no-fluorescence control (d) or negative (no virus) co-culture control (e).

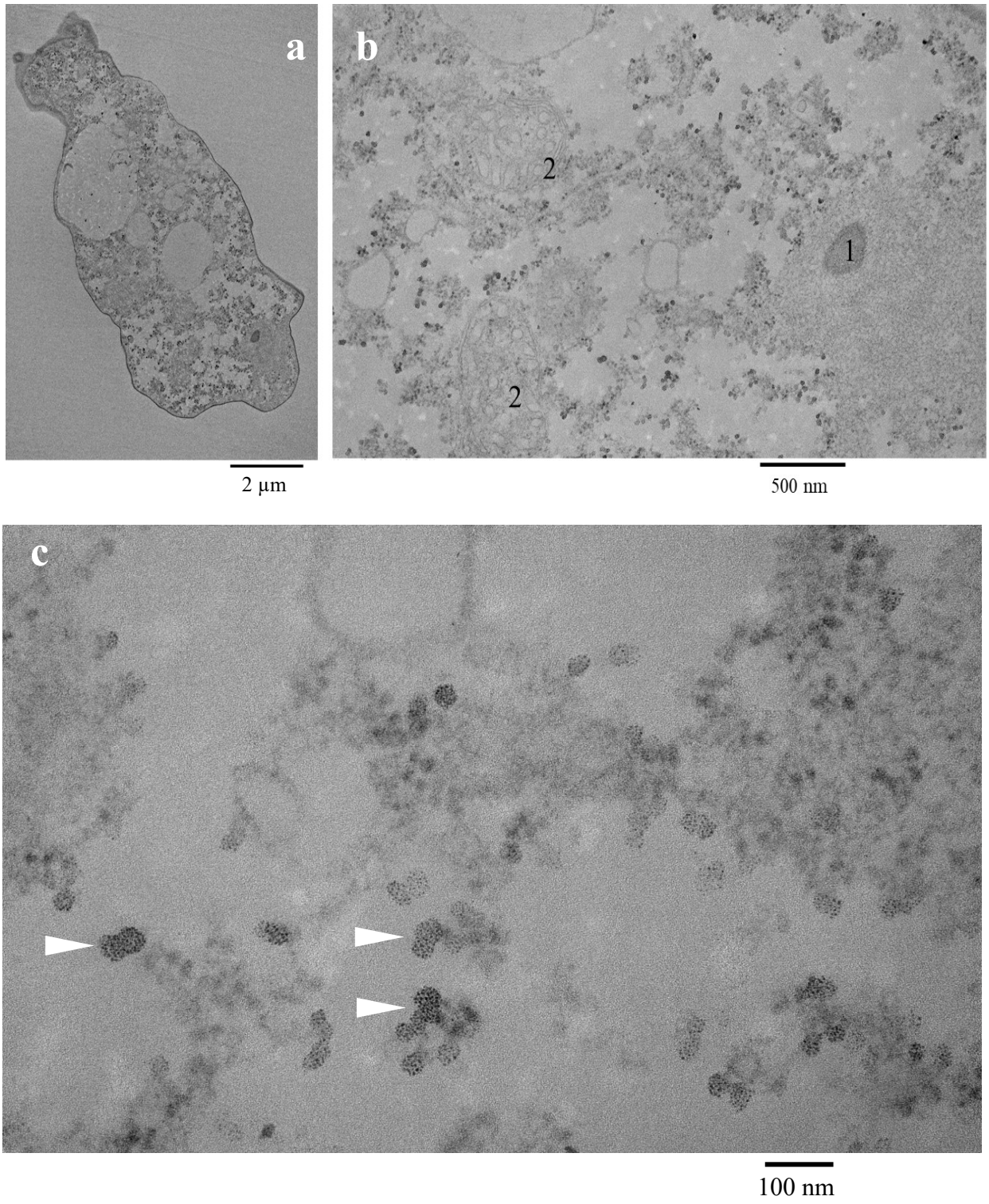


Figure 3.11 Transmission electron microscopy (TEM) of CVB5 in *V. vermiformis.* Samples were sectioned in 70 nm sections, contrasted, carbon coated (4.47 nm carbon fibers) and shown at different magnifications. In (a) the trophozoite was actively feeding prior fixation and had a

differentiated pseudopod. In (b) a section of the nucleus (1) and mitochondria (2) could be seen as well as virions appearing as dark spots. In (c) virus particles could be observed as having icosahedral-like shapes and forming small non-linear aggregates. CVB5 size ~ 30 nm in diameter.

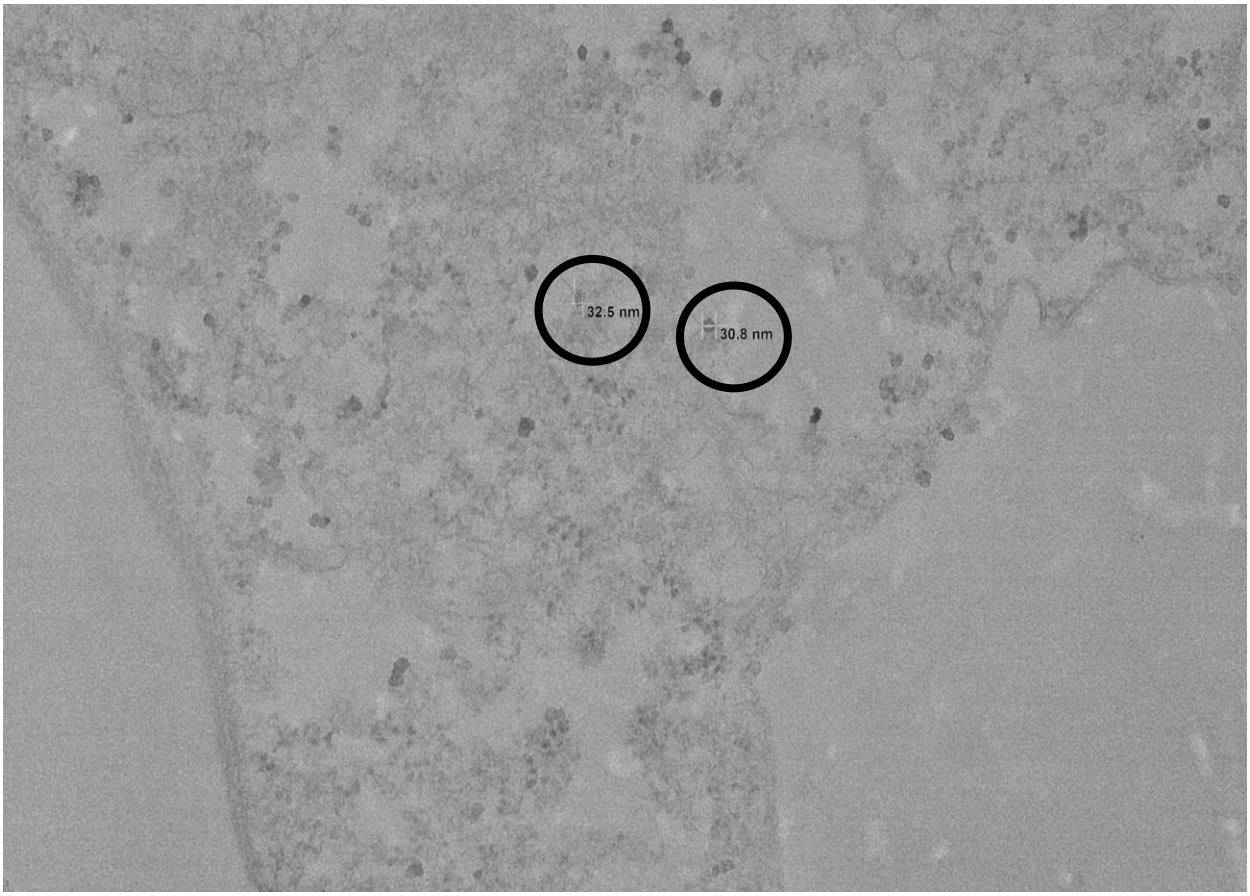


Figure 3.12 Typical transmission electron micrograph where virion size was measured and displayed by the Hitachi TEM software. The labeled examples are of 30 - 32.5 nm diameter and are within the expected size range for enteroviruses.

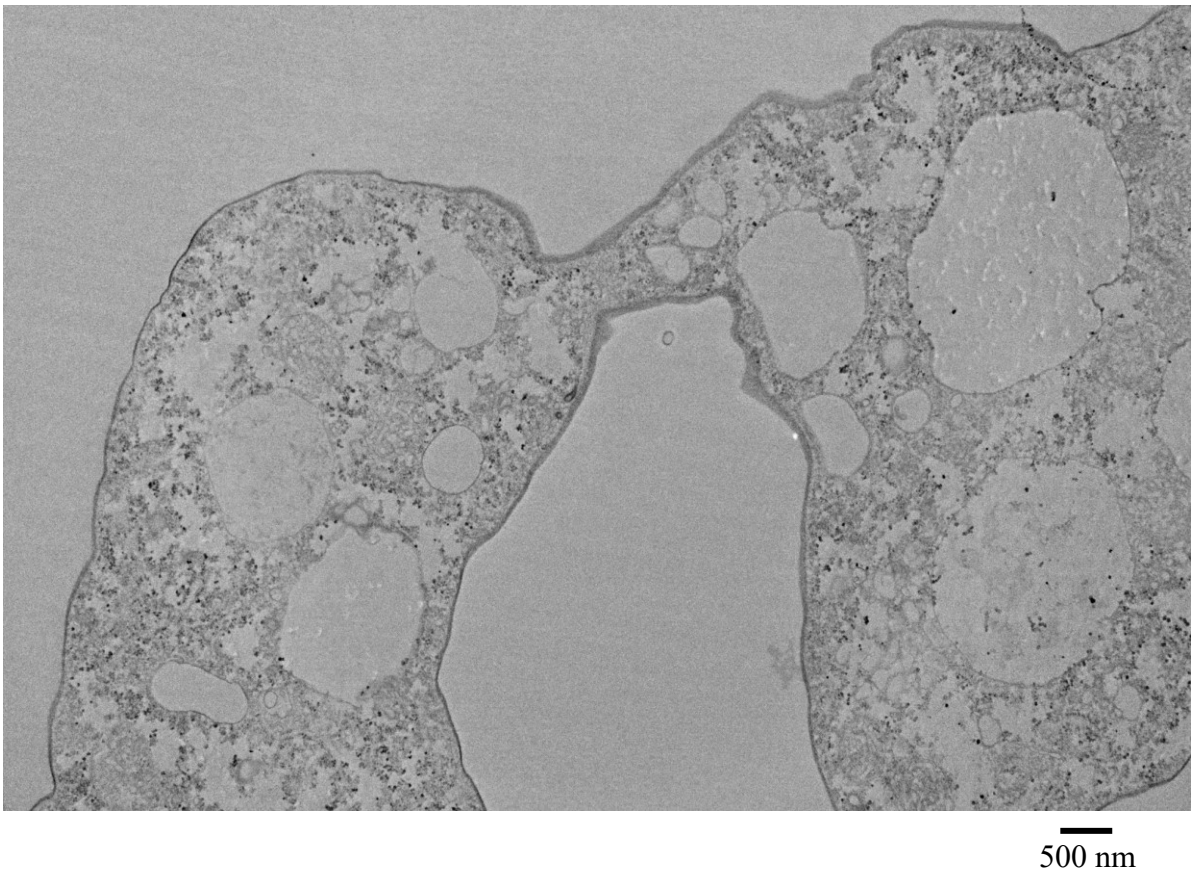
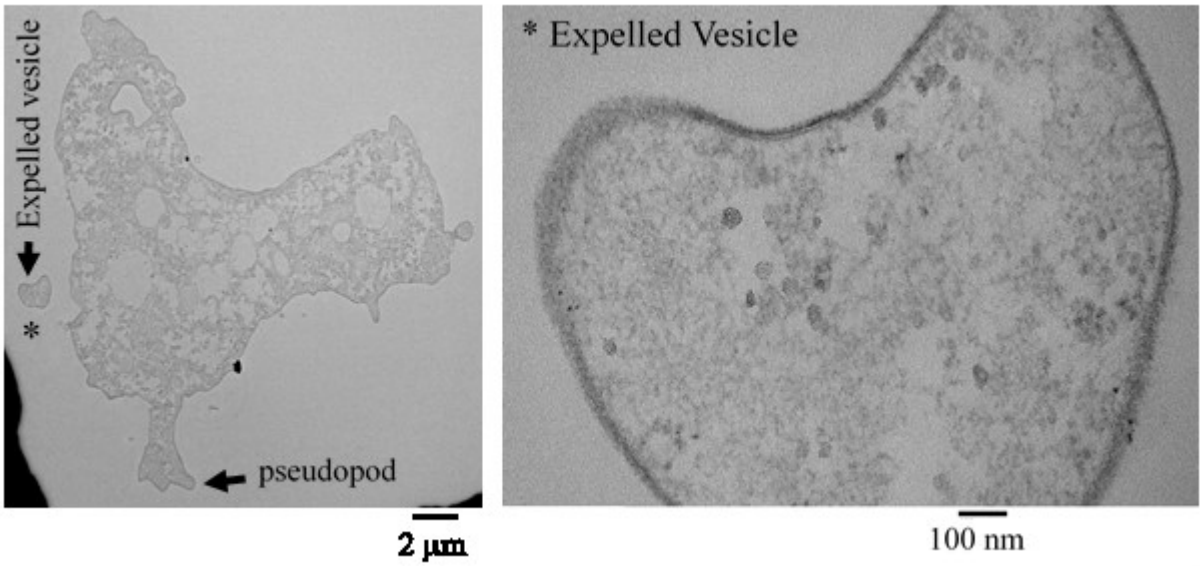


Figure 3.13 Virus-like particles observed in replicating *V. vermiformis*



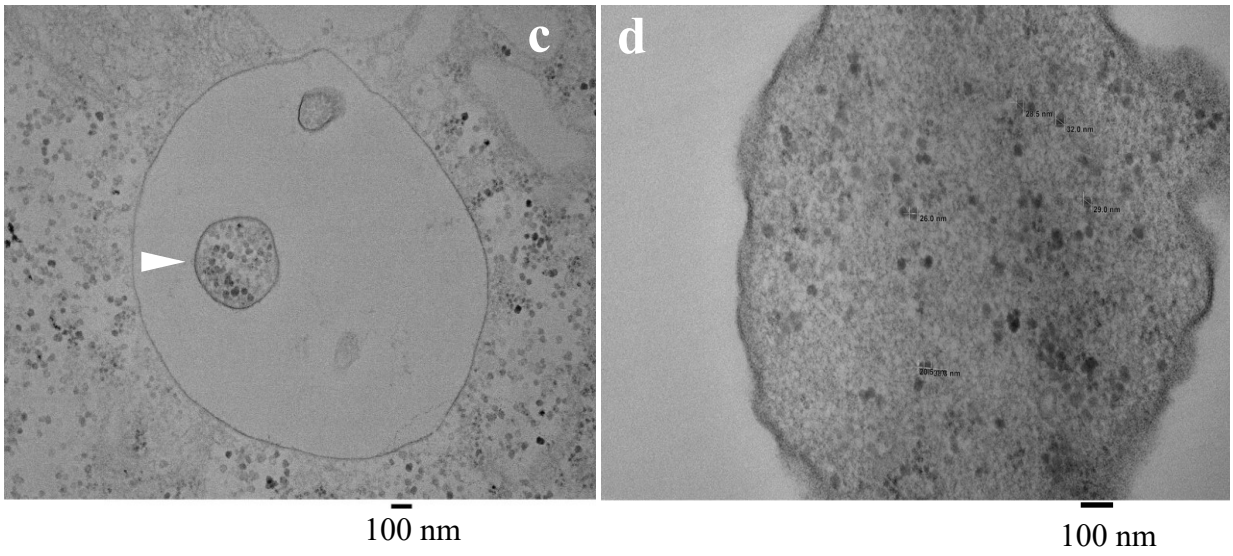
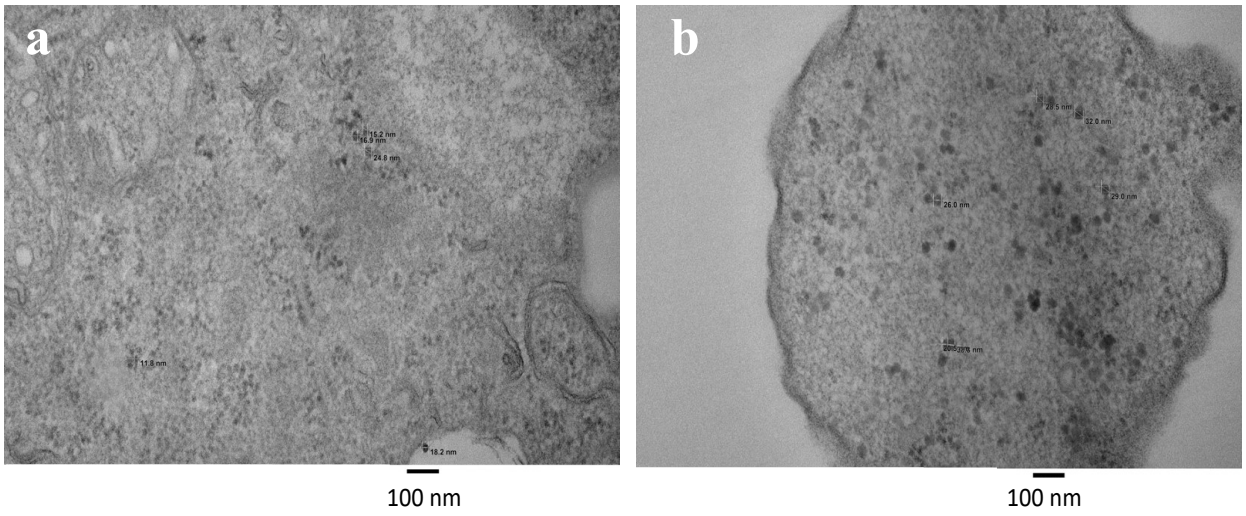


Figure 3.14 Example transmission electron micrograph of CVB5 in expelled *V. vermiformis* vesicles. In a) and b) CVB5 was found within a freshly expelled vesicle (EV) from a trophozoite form of the amoeba, with a differentiated pseudopod (P); in c) virions are contained within a vesicle inside what appears to be a contractile vacuole (CV), or another vesicular body, while in d) virions are again observed in an expelled *V. vermiformis* vesicle with their sizes confirmed by the Hitachi TEM software as ~ 30 nm in diameter.



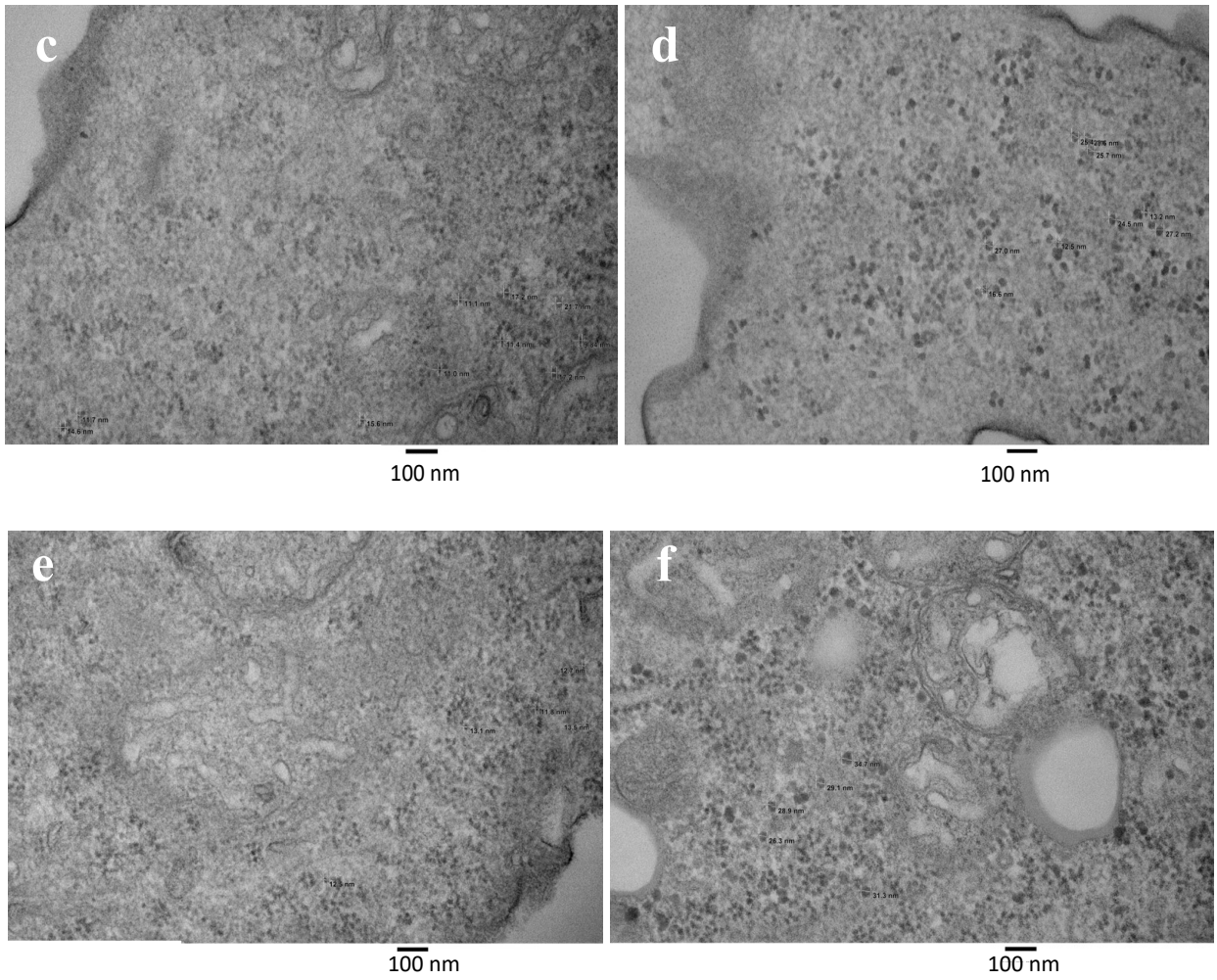


Figure 3.15 TEM comparison of *V. vermiformis* exposed to or unexposed to CVB5. Virus-particle size was measured with the Hitachi TEM software measuring tool for *V. vermiformis* exposed (b, d, f) to or unexposed (a, c, e) to CVB5. In b) the sizes of virus-like particles were compared also for an expelled vesicle (also shown in Figure 3.14).

3.5.6 Virus localization in *A. polyphaga*

Fluorescence signal was observed in *A. polyphaga* trophozoites and cysts, however, also in some of the cysts from the no-virus control co-cultures, particularly from the inner cellulose cyst wall of *A. polyphaga*, which may have some unspecific auto-fluorescence (Figure 3.16). *A. polyphaga* are notorious for rapid encystment as result of chemical exposure, [211,229,230] which could also result in the retention of fluorescent antibodies, however, the difference in fluorescence signal intensity between the negative control and the CVB5 co-cultured samples appeared to be significant. In

addition, there was a difference in the appearance of co-culture negative controls, which were stained and fixed as virus-containing samples, as opposed to the untreated no-fluorescence controls, which were not exposed to chemicals, therefore further confirming that the rapid encystment of *A. polyphaga* could interfere with some staining procedures as well. This was not observed for amoebae stained with a milder protocol (Figure 3.17).

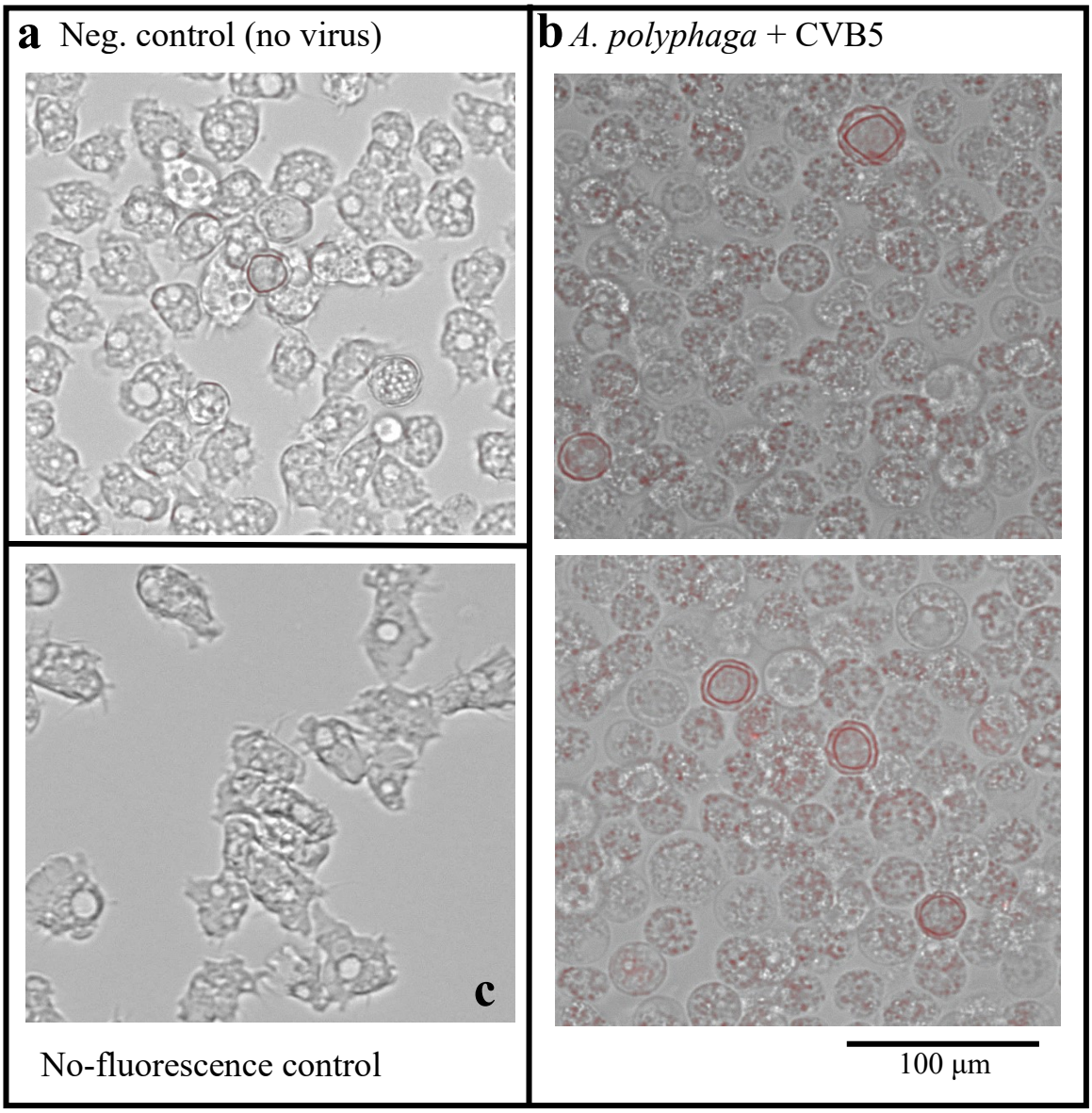
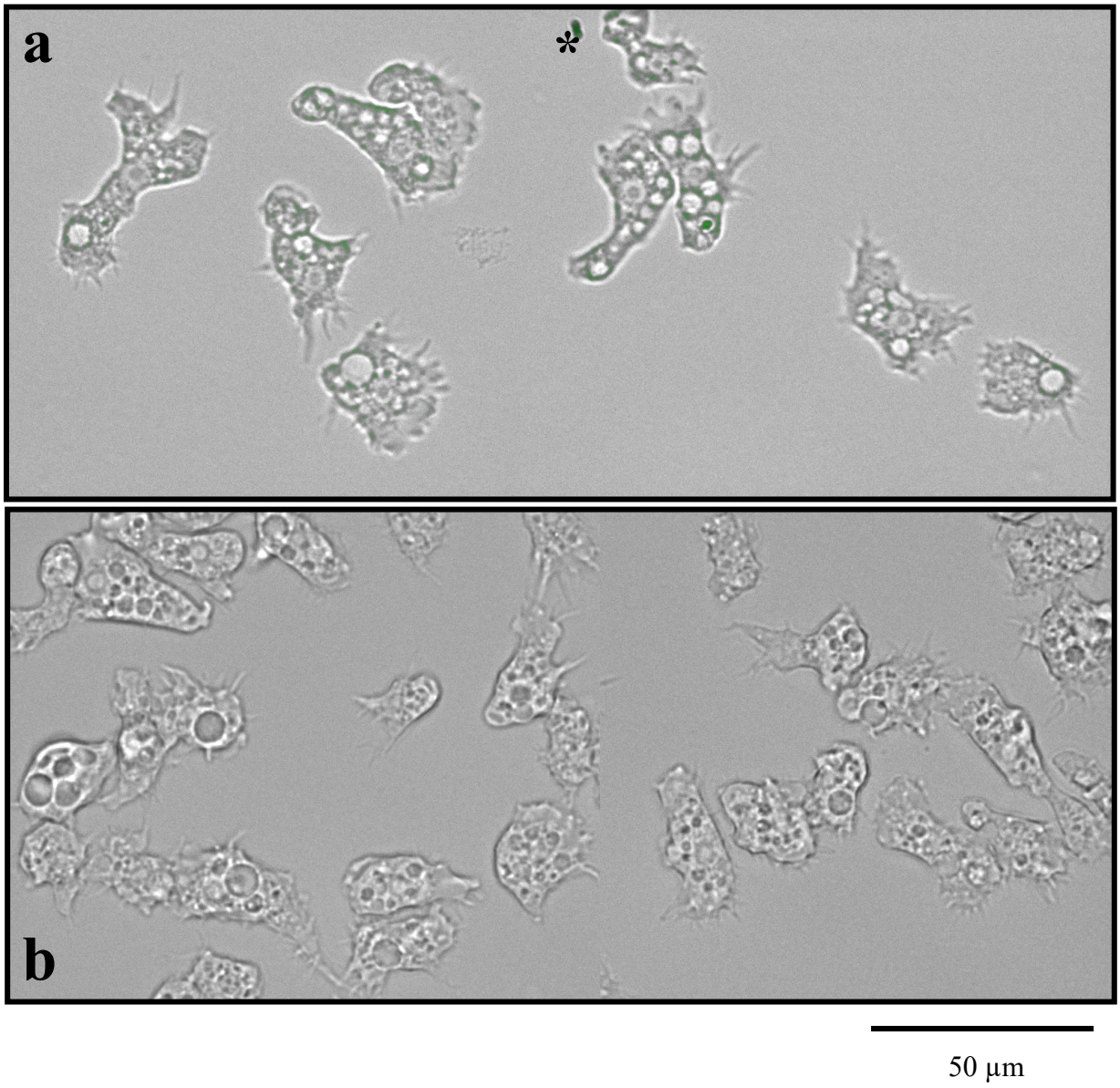


Figure 3.16 Double fluorescence staining of CVB5 in *A. polyphaga* trophozoites and cysts. The red signal represents Rhodamine-conjugated IgG-s. The negative control samples and CVB5-containing samples were subject to staining and fixation, while the no-fluorescence control included untreated amoebae.

The fluorescence signal associated with *A. polyphaga* pellets suggested either CVB5 internalization or tight adsorption to the surface of the amoebae (Figure 3.16 and Figure 3.17). To further explore the possibility for internalization, tests were also undertaken to examine the likelihood of *A. polyphaga* using CVB5 as a source of nutrient for growth (Figure 3.18).



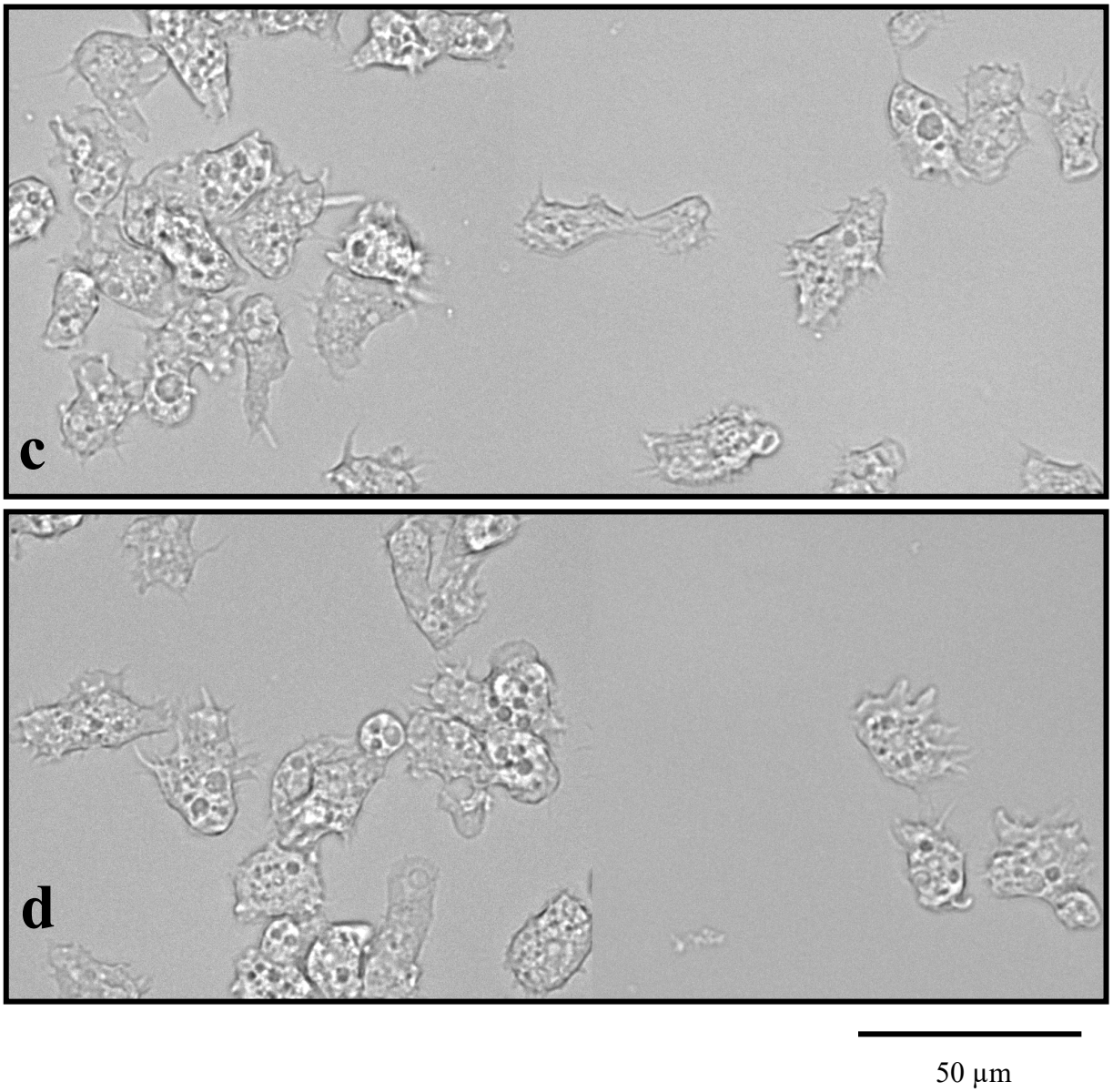
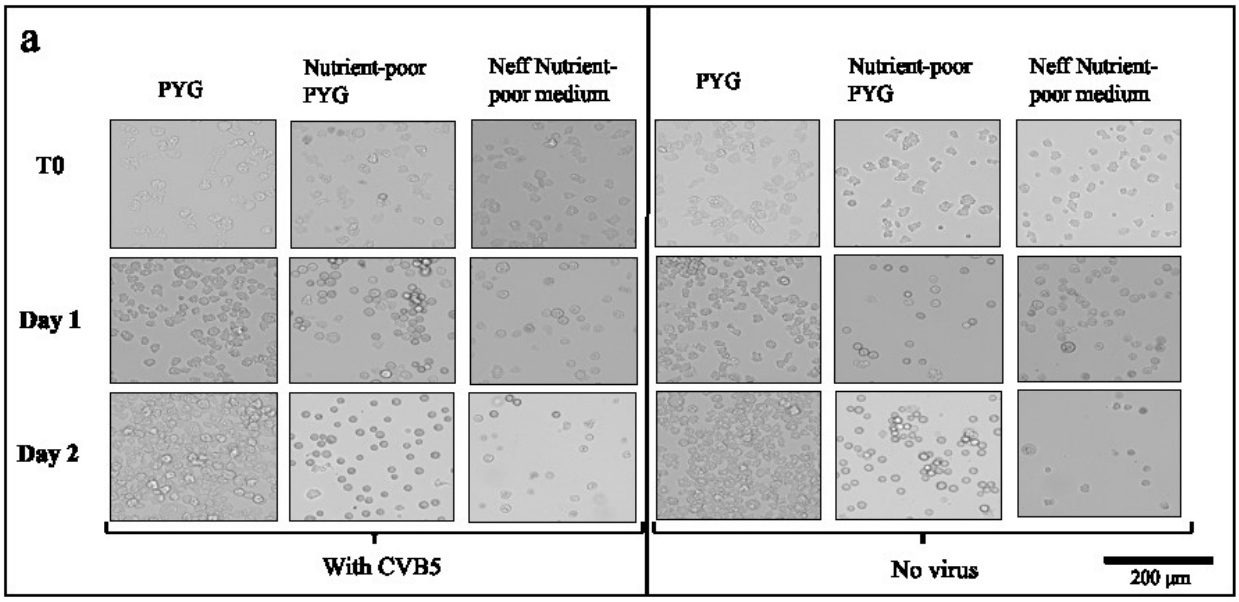


Figure 3.17: Fluorescence imaging of CVB5 in co-culture with *A. polyphaga.* MOI = 1,000; 1 dpi. Two photos shown in each section (a – d) out of 5 random fields per view. In a) CVB5 was stained and visualized as described here for *V. vermiformis* (milder staining as compared to Figure 3.16) and compared to negative controls (non-exposed amoebae) in b). Both co-cultured and non-CVB5 exposed amoebae, were also each stained with secondary antibody only (c and d respectively) to check for unspecific fluorescence. In a) * burnt computer screen pixel from the imaging system

3.5.7 Ability of *A. polyphaga* to use CVB5 as a growth nutrient

Co-cultured *A. polyphaga* with CVB5 in PYG were compared to nutrient poor media, which would require an alternative source of nutrition for amoebae to grow (such as the present virions). In the presence or absence of CVB5, at different temperatures, the appearance of *A. polyphaga* did not differ significantly in PYG (Figure 3.18). The same was observed for amoebae grown in PYG nutrient-poor medium, however, FLA grown in Neff's nutrient-poor medium differed in cyst size when they were grown at 37ºC in the presence of CVB5 for two days (Figure 3.18b). The larger cysts, compared to the no-virus controls, indicated that the amoebae were likely feeding and then encysted, as compared to the small cysts observed when amoebae continuously starved from the beginning. Despite this observation, however, it appeared that *A. polyphaga* could not gain significant nutrition from the presence of CVB5 alone, yet it is very likely that the amoebae could internalize the virus when starved and actively grazing.



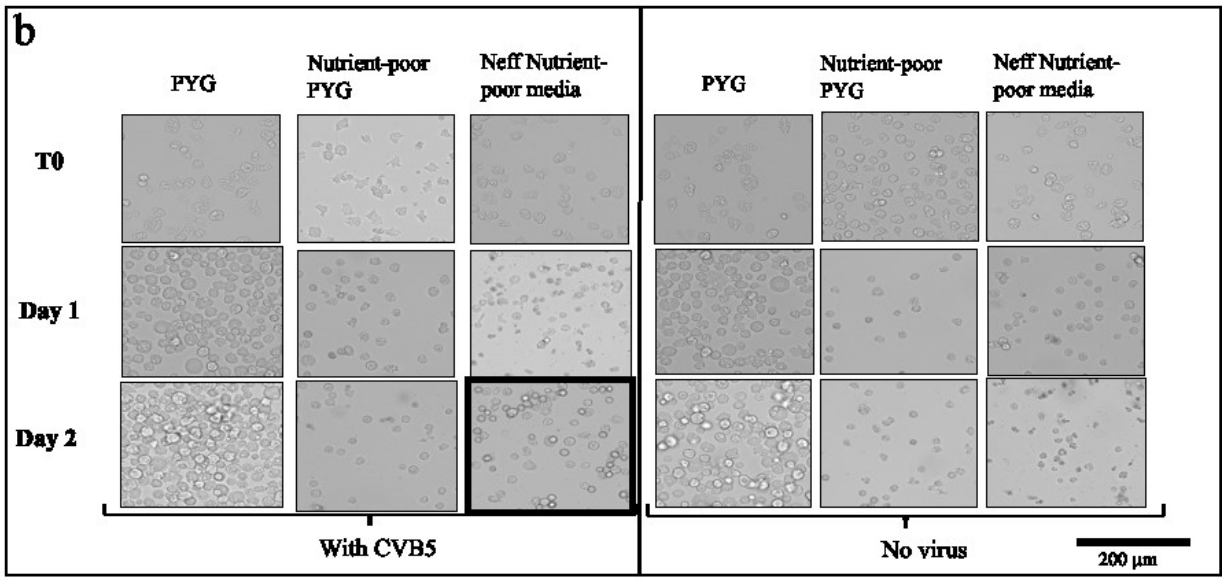
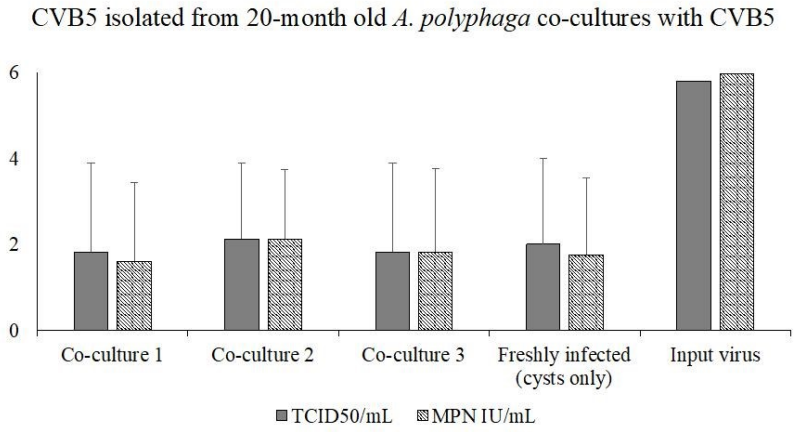


Figure 3.18 *A. polyphaga* in nutritious or starvation media with or without CVB5. The amoebae were grown at room temperature (a) or 37ºC (b) in the presence or absence of the virus for the duration of two days until complete encystment due to starvation.

3.5.8 Long-term CVB5 persistence in *A. polyphaga* cysts

In addition, CVB5 was also isolated from 20-month old mature *A. polyphaga* cysts (Figure 3.19). Infectious virus was detected from 10 out of 18 (~ 55%) of tested mature cyst suspensions, which were co-cultured with it 20 months prior. Because CVB5 was not isolated from all mature cyst samples, the plotting of the data as average values could be misleading, therefore the infectivity assays had to be analyzed including the raw data, especially when the emphasis is more on the presence or absence of infectious CVB5 within these long-term persisting cysts. Overall, a small amount of infectious CVB5 remained in association with mature *A. polyphaga* cysts for longer than one year, however a similar amount of CVB5 infectious titers were isolated from cysts of freshly co-cultured *A. polyphaga*, which were treated the same way as the older cysts (to ensure that in both cases all cysts were fully matured, and no trophozoites or intermediate forms were present). The fact that the encystment process involves nutrient and water expulsion, and the observed similarities between freshly-infected and older cysts, suggested that there was a significantly smaller chance of CVB5 persistence inside *A. polyphaga* cysts, however the

internalized (or adsorbed virus) associated with such cysts could remain so for an extended period of time (Figure 3.19).



	$TCID_{50}$/mL			MPN IU/mL		
Co-culture 1	0	0	200	0	0	120
Co-culture 2	100	100	200	51	51	120
Co-culture 3	0	200	0	0	120	0
Freshly infected cysts	0	200	100	0	120	51
Input virus (fresh)	5.8			5.96		

	Log10 $TCID_{50}$/mL			Log10 MPN IU/mL		
Co-culture 1	0	0	2.3	0	0	2.08
Co-culture 2	2	2	2.3	1.71	1.71	2.08
Co-culture 3	0	2.3	0	0	2.08	0
Freshly infected cysts	0	2.3	2	0	2.08	1.71
Input virus (fresh)	6.3×10^5			9.2×10^5		

Figure 3.19 CVB5 infectious titer associated with mature 20-month old thoroughly washed *A. polyphaga* cysts. Isolation of virus from cysts of each of three co-cultures was performed in triplicates (n = 3), where each data point replicate represents infectivity data obtained from quadruplicate serial dilutions. The error bars represent one standard deviation. The input virus values shown refer to the input virus used for the freshly co-cultured controls. For MPN data, from cysts vs. from input virus $P = 0.01226$ and among replicates of the same co-culture (fresh or old CVB5-co-cultured cysts) $P < 0.05$. For $TCID_{50}$ data, from cysts vs. from input virus $P < 0.05$ and among replicates of the same co-culture $P < 0.05$, based on a two-factor ANOVA.

3.6 Summary of findings

Infectious thermotolerant CVB5 could persist in association with *V. vermiformis* and *A. polyphaga* over time in liquid culture media. In both cases the virus retained its infectious potential over the duration of the experiments, and a much lower amount of infectious CVB5 titer was associated with FLA pellets, however the amount detected is still concerning given the low infectious dose

of coxsackieviruses. In addition, the virus could persist for long periods of time in association with *A. polyphaga* cysts. The virus was observed in all life stages *of V. vermiformis*, including expelled vesicles and did not cause any visible cytopathic effects in either *V. vermiformis* or *A. polyphaga*. These findings confirmed the association of infectious CVB5 with the FLA species most commonly reported in piped water systems (such as tap water and hot water tanks – *V. vermiformis*), as well as with the environmentally ubiquitous opportunistic human pathogenic amoeba *A. polyphaga*.

Chapter 4: Discussion

This thesis work demonstrates that in co-culture with actively feeding *Acanthamoeba* and *Vermamoeba* spp., an infectious clinical coxsackievirus B5 isolate, could persist and remain infectious for at least several days, and lesser numbers persisting in cysts for over a year. This is concerning, because both FLA species used here are reported in water supply systems, which are not normally stagnant, but amoebae accumulate in sediments/biofilms, and may excyst with trophozoites becoming active within as little as 24 h. [155,184,191,198–200] The ability of high-risk water contaminants, such as coxsackieviruses, to persist for prolonged periods of time in association with environmentally ubiquitous amoebae is concerning due to factors such as FLA persistence in hospital water systems, suggested links to nosocomial infections (especially concerning for coxsackievirus outbreaks in neonatal intensive care units, and even FLA persistence on a variety of surfaces and medical equipment). [22,23,44–46,56,158,154–156,160,176,193,209,210,217–222,262–264,302,303]

Previous pioneering reports have looked into the ability of *Acanthamoeba* spp. to internalize viruses, including coxsackieviruses, however, such research had important limitations. All of these reports were performed in cell-culture media instead of optimal amoebae growth media. [289–291] This is of great concern because it is unknown whether human viruses will be able to use receptors to infect environmental protozoa in the classical sense of infection, and as the results of my research suggest, the virus did not cause any visible cytopathic effects on either *V. vermiformis* or *A. polyphaga*. In addition, the results presented here, demonstrate that CVB5 could be packed in *V. vermiformis* vesicles, and expelled in the surrounding liquid environment, while the amoebae remain replicating, and able to form intact pseudopods. CVB entry in some cases interferes with actin cytoskeleton rearrangements and its efficient replication interferes with eukaryotic cap-dependent translation. [93,94,148] It is therefore more likely that the virus is internalized prey for the amoebae, which can be ingested through phagocytosis or pinocytosis (or both), since neither the shape, nor the ability of the amoebae to grow over time were impacted by the co-occurrence of CVB5. In addition, the ability of *V. vermiformis* to expel internalized virions via expelled vesicles (Figure 3.14), suggests that the amoeba could eliminate indigestible virus particles. The size of these particles was measured with the Hitachi TEM software as ~ 30 nm diameter, and such

particles were not observed in *V. vermiformis* unexposed to CVB5, while other small granular structures (possibly ribosomes) were observed in both exposed and non-CVB5 exposed amoebae. Furthermore, as evident from Figure 3.11, the virus-like particles appeared to have icosahedral-like shapes and they formed non-linear aggregates (seemingly attached to one another's edges), which in combination with the dotted-appearance after contrasting (likely due to capsid depressions) and the absence of such structures in non-CVB5 exposed FLA, confirmed that these internalized 30 nm structures were indeed virions.

The findings presented in this thesis work demonstrate that amoebae do not grow efficiently in classical cell-culture media, and therefore making accurate conclusions involving pinocytosis is not possible in sub-optimal amoeba growth media due to the reliance of actively feeding patterns for the FLA to be able to efficiently internalize virions from surrounding growth media. In addition, previous findings, which have reported contradictory conclusions, have used different assays to demonstrate virus persistence in amoebae (either non-quantitative PCR or $TCID_{50}$), as well as fluorescence microscopy, where, given the virus-amoebae ratios used, considerable auto-fluorescence was likely. [289–291] The findings presented here demonstrate that rigorously stained *A. polyphaga* is more likely to encyst, and its cysts may autofluoresce (Figure 3.16), while *A. polyphaga* stained with only antibody set (and therefore treated more gently) appeared to remain in trophozoite forms (Figure 3.17) with no observed autofluorescence, which could also be due to the choice of fluorophore, since *A. polyphaga* cysts walls appeared to slightly fluoresce in red, and therefore the choice of FITC-conjugated secondary IgG seemed to be the better option. Such optimizations in terms of treatment, staining, and optimal growth media have not been reported in previous findings where the topic of virus-*Acanthamoeba* spp. interactions has been addressed. [289-291]

The current findings demonstrate that while indeed some assays can be problematic for some environmental protozoa, such as *A. polyphaga,* even when the same type of assay is used (such as the estimation of virus infectious titer only), results could differ if calculated by different formulae (*e.g.* MPN vs. Reed-Muench formula) as seen from the different statistical values shown in Figure 3.5 where the ANOVA analysis was done for the exact same data set, with the only difference being the choice of formula to calculate virus infectivity. Whenever possible, in this thesis such

studies were also combined with a highly-sensitive RT-qPCR assay, and again, such a thorough analysis - quantifying changes in the amount of virus particles it takes to comprise an infectious unit (Figure 3.6), have not been reported with FLA. In combination with the first-time observations of a high-risk human virus, such as CVB5, packed within expelled tap-water amoeba vesicles (*V. vermiformis*), my research addressed existing knowledge gaps, while providing a guideline for methodologies that could be used for future such studies. Both molecular and cell culture assays have limitations - PCR assays can detect infectious and inactivated virions, while infectivity assays alone can estimate the number of infectious units, but not the number of virions needed to comprise one infectious unit. Therefore, it is always better to use different methods to address the same research question.

Contradictory reports often result in failure to follow up on the topic, and as a result, important knowledge gaps remain poorly understood, such as the persistence of high-risk enteric viruses in ubiquitous free-living protozoa. The findings reported here successfully address the initial question about persistence of a clinically infectious enteric virus in relevant FLA strains. The results also demonstrate the importance of strictly consistent amoebae growth conditions, where optimal choice of growth media is not the only problem, but where extensive washing steps could also interfere with proper virus detection from liquid co-culture samples, as actively feeding amoebae may contain, or even expel virions in vesicles (as demonstrated here for *V. vermiformis*). While washing steps can be important for experiments involving immortalized cell lines, in which virions could replicate rapidly, and input virus needs to remain consistent for successful comparison among research samples, it is also important to emphasize the differences between free-living environmental protozoa and immortalized lab-adapted cell lines. Therefore, assays need to be adjusted accordingly depending on the organisms used. This way important knowledge gaps could be addressed with more consistent findings, which could later be important for risk assessment and other studies on pathogen monitoring.

Type B coxsackieviruses, not only target the most vulnerable portion of the population but could also cause illness in healthy adults. [75,91] Internalization by FLA could significantly impact the risk assessment of these and other similar high-risk viruses. Within water biofilms, for example, the amoebae actively feed on bacteria, some of which are human pathogens, such as *L. pneumophila*.

[36,154–156] In the case of co-internalization of pathogens inside FLA, the subsequent dispersion from water systems could lead to multiple infections. In some cases, concurrent human exposure to protozoa, in combination with other pathogens, could elevate the infection risks. [244] FLA-bacteria interactions provide selection for phagocyte resistance, and therefore it is important to note that other eukaryotic phagocytes, such as macrophages, play an important part of primary immune defenses through TLR receptor signaling and nitric oxide production. [147,148] Multiple infections could compromise macrophages and lead to elevated risks of infection. This is an example of FLA interference with dose-response relationships of other microorganisms. In addition, some amoebae, such as *Naegleria fowleri* and *Entamoeba histolytica,* reportedly also produce nitric oxide, which is suggested to play a role in their respective pathogenicity. [303–305] Microorganisms internalized by such amoebae could be subject to selective pressure for nitric oxide resistance and this may have an effect on subsequent susceptibility of human macrophages to infections. It is currently unclear whether other FLA species are able to produce nitric oxide, however this may also be species-specific. FLA are highly diverse (like human viruses and pathogenic bacteria), therefore FLA-virus interactions could also be highly diverse, thus emphasizing the importance of investigating such complex interactions in contaminated waters as sources of environmental interference with virus management in systems.

The results reported here demonstrate that CVB5 can be found in all life stages of *V. vermiformis* (trophozoites, cysts, and expelled vesicles). CVB5 could persist as infectious virus titer in co-culture with the amoeba without causing any visible cytopathic effects. The virus persistence in association with *A. polyphaga* also did not cause any visible cytopathic effects. In addition, *A. polyphaga* may be able to utilize the virus as a source of nutrient, however, the virus is unlikely to provide sufficient long-lasting nutrition for growth of the FLA (Figure 3.18). This only emphasizes the fact that FLA in the environment do rely on their bacterial prey for adequate nutrition, however the co-occurrence of FLA with human viruses in water biofilms does make it likely that virions could also be internalized, more likely as accidental prey, in addition to bacteria, and possibly even bacteriophages. In this regard, these novel findings support the conceptual model of FLA interference with accurate enteric virus risk assessment on different levels, especially in the event of multiple infections resulting from FLA dispersion through water systems.

In addition, actively feeding amoebae migrate and graze on water biofilms, therefore they could continuously internalize virions and possibly also pack them, concentrate them, and release them in expelled vesicles, which has previously been observed for pathogenic bacteria. [154,256,261] This could be a contributing factor to virion or virus-particle concentration, which has been reported to occur within wetland and piped water biofilms. [20,306] It is currently not well understood whether FLA could play a role in this, however the presented results demonstrate that internalized CVB5 could be packed and expelled via vesicles of the tap water amoeba *V. vermiformis* into surrounding liquid media.

Another concern in this regard is the recognition of CVB as environmental factors for Type 1 Diabetes. [69,76,92,147] It is currently not known whether FLA could play a role in this. The viruses are especially associated with monocytes of T1D patients and have been proposed to utilize migratory immune cells in "Trojan horse"-like scenarios for spreading across epithelial surfaces, in addition to being able to cause productive infections in macrophages. [92,147,148] The observation that a clinical CVB5 isolate could remain associated with waterborne FLA (as demonstrated here), especially for prolonged periods of time in association with mature cysts, raises the important concern for eventual exposure to human macrophages, which is likely to involve multiple infectious agents and is therefore likely to compromise macrophage defenses. In addition, *Acanthamoeba* spp. can degrade host immunoglobulins, cytokines and complement proteins. [213,216] This further elevates health risks associated with FLA-internalized pathogens, especially for immuno-compromised individuals. [160] There is also a possibility that infectious doses and dose-responses associated with internalized virions may not be directly affected, however on the other hand, dose response relations of other concurrent pathogens may be affected. This is largely speculative and emphasizes the importance of further investigations in the field, especially due to the fact that, like FLA-bacterial interactions, FLA-virus interactions could also be highly specific and diverse, depending on the species and strains involved. [242–246,292]

The findings shown here demonstrate that CVB5 did not cause any visible cytopathic effects in either *V. vermiformis* or *A. polyphaga,* however, previous preliminary reports have shown that polioviruses can lyze some amoebae, such as *Amoeba proteus.* [289] This raises another important question for lytic viruses, which would need to lyze host cells in order to complete their infectious

cycles. Lyzing FLA hosts may deprive internalized virions of potential environmental niches for persistence and shelter from disinfection. Coxsackieviruses have been reported as either highly lytic or latent, depending on the cell cycle status and cell type. [142,143] It is therefore important to investigate interactions, such as those of FLA and human viruses, from different angles (evolutionary diverse species involved, such as the amoebae species used in this research).

The research described here did not show any conclusive evidence for viral replication in co-culture with the amoebae, and in fact, the internalized virus fractions had a decline in virus infectivity, despite the persistence of some infectious virus over time (Figure 3.3 – Figure 3.6). This is an important aspect of FLA-virus interactions to consider because viruses, such as enteroviruses, are prone to high mutation rates. [49] In natural hosts, lethal mutations are eliminated from the viral gene pool, however in alternative hosts, such as environmental protozoa, lethal mutations may not be selected against, which could have an effect on the tropism and infectivity of the virus (hence again potentially interfering with its infectious dose as result of environmental exposure to amoebae). CVB replication is observed to interfere with cap-dependent eukaryotic translation, therefore this is one means of cell injury. [93,94] CVB infections could also involve actin cytoskeleton rearrangements as another way to affect infected cells. [148] The TEM images shown here demonstrate that virions could be found in healthy *V. vermiformis* cells, which were capable of replicating successfully and forming healthy pseudopods. In addition, undigested virions were packed and expelled by the amoebae through vesicles, which in combination with growth and encystment studies, suggested that CVB5 is unlikely to cause productive infection or any visible cell injury in the amoebae, and therefore it is not very likely that the virus could replicate in the FLA species tested here, but rather persists inside the amoebae and their vesicles. Such persistence could be beneficial to the virus, in terms of preserving its genetic integrity from acquiring unnecessary mutations, while still having a potential shelter from biocides or other environmental factors. Alternatively, however, digestion by FLA could damage virus epitopes required for cell receptor binding, and this is also a likely scenario given the observations that internalized virions had reduced infectious potential as compared to total co-cultured virus or virus unexposed to amoebae. Overall, it is not yet possible to conclude whether enteric viruses could actually benefit from internalization by FLA, other than the likely protection from disinfectants and environmental stressors, such as heat (*e.g.* in hot water tanks).

In terms of disinfection, an interesting fact about *Acanthamoeba* spp. is their ability to produce pheromones and signal danger to neighboring cells. [161,180-182] This way the amoebae can form dormant cysts way before being exposed to an actual biocidal agent. *V. vermiformis,* on the other hand, is notorious for resisting a variety of disinfectants used in dental waterline units. [161,184,192,193,301] When the amoebae excyst, they usually have a large number of autophagosomes, which is an important link to the reported ability of CVB to utilize such vesicles throughout its infectious cycle. [96,170] While this is unlikely to result in FLA damage from the virus, as no evidence of that was observed here, it could help explain why some virions were packed in vesicles targeted for expulsion or destruction, as seen from TEM images. It is not clear however, whether these virus-containing vesicles are trapped inside a food vacuole, autophagosome, lysosome, or another multivesicular structure since this is not clear from the TEM images alone.

Furthermore, drinking water distribution pipes could be exposed to enteric virus contaminations through contaminated soils, therefore the association of CVB5 with *A. polyphaga* (which is common in humid soils) is additionally concerning, in the events of water distribution pipes being exposed to soil contaminations via intrusion points from pressure changes in pipes. [34,162]

Another important aspect of FLA, when it comes to their likelihood to interfere with virus removal, is the fact that amoebae, such as *A. polyphaga*, contain large amounts of glycogen and complex polysaccharides, [160,176,227,228] therefore the presence of high number of sugars could mean a vast range of pH fluctuations between cytoplasm and food vacuoles (or other vacuoles/vesicles the virus may be localized in). As a reminder, virus exposure to fluctuating pH in the environment could interfere with capsid charges and therefore with physical removal of virions from waters. [124] Complex FLA metabolites could also act as PCR inhibitors, therefore further interfering with virus detection. Depending on the virus species, digestion of epitopes may also interfere with virus culturability from environmental water samples, therefore virus internalization by FLA does have the potential of interfering with risk assessments on multiple levels as suggested by the conceptual model (Figure 1.7). Outbreaks of waterborne infections in developed countries often occur due to lack of understanding of short-term events in systems. [34] As a result, failure to sufficiently disinfect drinking water supplies could lead to waterborne enteric outbreaks, in which cases both

enteric viruses and FLA could be present. [10,307–309] Proper disinfection practices, however, need to be constantly monitored and evaluated, especially due to chances of multiple levels of interference from poorly understood complex microbial interactions, and likely virion protection within amoebae (as seen with bacteria). [309]

Pioneering research on mixed matrices could be challenging, which is also observed with experiments involving *A. polyphaga.* Existing reports on virus-FLA interactions currently provide only preliminary aspects, and combined evidence of virus internalization, persistence over time, and effect on the amoebae was missing, which is especially important for assays performed in sub-optimal amoebae growth media, or in cases where different studies have used different methods (such as non-quantitative PCR, or $TCID_{50}$ assays only). [283–291] In some cases fluorescence-based assays exhibit significant unspecific fluorescence given the virion-protozoan ratios used. [289] Overall this results in increasing knowledge gaps, as evident from failure to follow up on the topic. The *A. polyphaga* co-culture results presented here, and difficulties with probe-based assays as opposed to *V. vermiformis* experiments, were consistent with difficulties observed in previous reports, however the use of different infectivity assays provided insight on virus infectious potential and could be useful for other FLA-virus studies where sensitive PCR methods may be challenging. In addition, the calculation of percent specific viral infectivity, shown here for co-cultures with *V. vermiformis,* is a useful method to apply, whenever possible, to other FLA-virus studies for the most accurate assessment of virus persistence in co-culture with amoebae over time.

In conclusion:

The presented results strongly support the thesis that an infectious clinical isolate of CVB5 could persist over time in co-culture with relevant FLA species isolated from water systems, and therefore the likelihood of FLA-virus interactions being important health hazard for high-risk virus pathogens, representing novel pathways currently not evaluated in microbial risk assessments. This health hazard could occur at multiple stages of the water (re-)use cycle (Figure 4.1), therefore FLA-virus interactions and associated risks have a broad significance, which needs to be investigated further. In addition, useful methodology was described, which could be applied to other virus-FLA interaction studies.

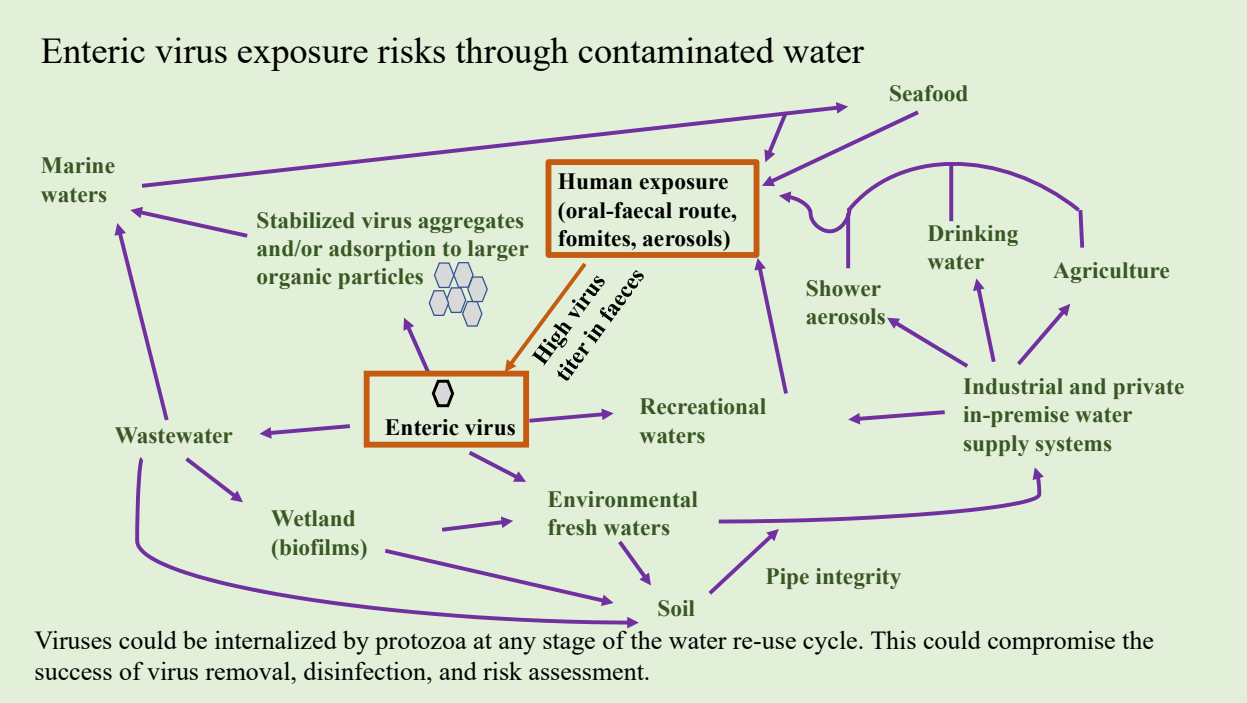


Figure 4.1 Enteric virus exposure risks throughout the water re-use cycle

Future directions:

Exciting aspects of the research, where follow-up is seen as a priority, include the investigation of FLA-virus interactions involving a variety of species and strains. In addition, efficacy of virus detection, removal, and disinfection, in the presence or absence of amoebae (cysts, vesicles, trophozoites) needs to be investigated further, together with the possibility for multiple human pathogens co-existing within FLA.

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