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Screening for viruses using unicellular eukaryotes as hosts

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Master's thesis in Biology

BIO-3950, November 2023

Acknowledgements

Throughout this academic journey, I have been humbled by the strength, guidance, and wisdom granted to me by the God Almighty. It's been a journey full of ups and downs, but I'm grateful for every moment. Without His blessings, I would not be where I am today.

I am truly grateful for the unending support and guidance from my supervisors, Associate Professor Gabriel Magno de Freitas Almeida and Professor Klara Stensvåg. Their unwavering commitment and patience have been fundamental in the successful completion of this work, and I humbly extend my heartfelt appreciation to them.

I owe a debt of gratitude to my parents, Asif Ernest Gill and Dilshad Akhtar, and my brother, Arun Asif, for their continuous love, encouragement, and sacrifices that made it possible for me to pursue this degree. Your unwavering support has been my constant motivation.

I would also like to express my thanks to my friends, including Waleed Abbas, who have stood by me with their encouragement and support during the highs and lows of this journey. Your belief in me kept me going.

Lastly, I am thankful to the entire Marine Bioprospecting research group I have been a part of, especially for the camaraderie and collaborative spirit that has enriched my academic experience. This achievement would not have been attainable without the collective efforts and contributions of all its members.

Tromsø 15.11.2023

Zaroon Ricky

Abstract

This master's project delves into the microbial diversity of the Arctic region, with focus on the isolation and characterization of a yeast of the *Cystobasidium* genus and likely viruses capable of infecting it. The research also shows preliminary results pointing at the movement and chemotaxis capabilities of the yeast, presenting findings that have the potential to shape our understanding of microbial interactions in marine environments. A new species of *Cystobasidium* was identified. In addition, *Cystobasidium* was used as unicellular eukaryotic host target for viruses from a diverse sample collection obtained from Tromsø's surroundings and other regions in the Arctic. Giant virus isolation attempts utilizing *Acanthamoeba* as hosts was also performed as comparative analysis of a new artic host. Giant virus isolation efforts yielded the discovery of potential infectious agents, particularly in a sample from a deep-sea vent, showcasing notable structural variations in electron microscopy. The necessity for full genomic sequencing to confirm whether our *Cystobasidium* is a new species and to prove the viral nature of the isolated agent is emphasized throughout the study, which concludes with a contribution to enhance understanding of microbial interactions in marine environments, setting the stage for further exploration and discoveries.

Abbreviations

µl	Microliter
µm	Micrometer
APMV	*Acanthamoeba polyphaga mimivirus*
CPE	Cytotoxicity effect
ICTV	International committee on taxonomy of viruses
ITS	Internal transcribed spacer
mg	Milligram
ml	milliliter
NCBI	National Center for Biotechnology Information
NCLDV	Nucleocytoplasmic large DNA viruses
NNA	Non-nutrient agar
ORF	Open reading frames
PAS	Page's modified Neff's amoeba saline buffer
PYG	Peptone yeast extract glucose broth
TMV	Tobacco mosaic virus

Table of Contents

List of Tables

List of Figures

1 Introduction

Microorganisms, commonly known as microbes, constitute a diverse group of microscopic living entities that inhabit various ecosystems and exert profound influences on biological processes. Their ubiquitous presence, rapid replication rates, and metabolic versatility make them fundamental to the functioning of ecosystems. Microbes can be classified into two main categories based on their cellular structures, namely prokaryotes and eukaryotes. While viruses are also part of microbiomes, they differ from other microbes for not having cellular structures and being obligate intracellular parasites. Microbial significance in various aspects of human life has been recognized including agriculture, biotechnology, medicine, and environmental sciences (Madigan et al., 1997).

Unicellular eukaryotes refer to microorganisms that comprise a single cell and belong to the domain Eukarya. They are distinguished by possessing a true nucleus, membrane-bound organelles, and a more intricate cellular structure relative to prokaryotic cells. Amoebas and yeasts are two prominent instances of unicellular eukaryotes (Campbell & Reece, 2005).

Extensively studied on amoebas are available, amoebas are unicellular eukaryotic microorganisms that exhibit a unique mode of movement called "amoeboid motion." These tiny organisms use cytoplasmic projections called pseudopods to move around and capture their food. Amoebas are omnipresent in aquatic environments and soil, and they are also known to be human parasites. These organisms feed by engulfing food particles through phagocytosis, where they surround the particles with their pseudopods. Due to their importance in various fields, such as biology, microbiology, and medicine, amoebas have been extensively researched, resulting in a better understanding of their ecology, genetics, and physiology (Cavalier-Smith, 1993).

Yeasts are a type of single-celled eukaryotes that belong to the kingdom Fungi. They play a crucial role in fermentation and are widely utilized in diverse industrial and culinary applications, such as baking and brewing. Yeasts propagate themselves through a process called budding, and they usually display a round or oval shape. While asexual budding is their main mode of reproduction, they can also reproduce sexually under specific circumstances. These organisms are everywhere in nature and can be found in various environments, including soil, plants, and the human body. Certain yeast species have significant biotechnological

applications in industries such as food and beverage production (Goffeau et al., 1996; Piškur et al., 2006).

1.1 Viruses

1.1.1 Conventional and historical context

Until recently sterilizing of liquids was done by using 0.2μm filter papers as an alternate of heating, which is an effective way to filter out bacteria and other microbes (Madaeni, 1999). Viruses can pass through microporous filters i.e., Chamberland-Pasteur filter which was used to filter the first virus. Tobacco mosaic virus (TMV) was the first virus ever discovered by a Russian scientist Dimitry Ivanovsky in 1892 when he observed that filtrate of the sample contains some kind of disease causing agent (Jerome et al., 2015). TMV was not culturable in absence of cells, not visible under light microscope and small enough to challenge the germ theory which recently accommodated bacteria so well (Van Loon, 2002).

Factors strengthened the idea that these are some type of non-living particles. On the basis of virus discoveries, how they reproduce, and advancement in electromagnetic imaging which helped take first image of TMV in 1939 (Kruger et al., 2000). During this period study of bacteriophages, viruses which infect bacteria, helped to come up with biological principles and gave the foundation for Lwoff's criteria in late 50s, which clearly distinguishes the cells from viruses, afterwards these criteria were updated in 1966 (Morange, 2005). The criteria comprised 5 distinguishing features separating viruses from cells: microorganisms contain nucleic acid in the form of DNA and RNA, translational machinery, possess enzymes for metabolic activity, reproduce with binary fusion. In contrast, viruses lacked all reproductive features, it only had either RNA or DNA which was required to reproduce. Lwoff's set criteria persisted for another of 50 years until 2003 when very first giant virus was found (Claverie & Abergel, 2012).

The lack of metabolic activity and lack of translation machinery explains the parasitic behavior of viruses (Gale Jr et al., 2000). One can compare viruses with the obligatory intracellular parasitic bacteria (*Rickettsia*) or symbiotic bacteria (*Buchnera*). Reductive evolution of bacteria has removed most of the metabolic functions in these intracellular bacteria. Viruses possess enough metabolic activity to keep up with the requirements to maintain typical cellular functionality for example energy transduction (Bokhari et al., 2020). With the discovery of giant viruses the boundaries to classify bacteria and viruses became unclear. This groundbreaking discovery in the field of virology has ignited debates. Just this question what

is the origin of viruses? brought in new questions of what is a virus? And what is their place in biosphere? The emergence of giant viruses intertwined with these historical questions (Lwoff & Tournier, 1966; Needham et al., 2019).

1.1.2 Diversity and taxonomy of viruses

Structural diversity in viruses is quite limited. Typical structure of symmetrical protein capsid made of repeating subunits of protein called capsomers. Occasionally, a protective layer made of two layers of fat molecules can surround it, retained from the host cell. Several morphologies have been observed to date. However, significantly diverse but the structural parameters of capsomers are quite limited. The two most prevalent shapes observed in viruses are those that are rod-shaped (with capsids exhibiting helical symmetry) and those that are spherical (displaying icosahedral symmetry)(Louten, 2016).

The Binomial nomenclature help classification of cellular organisms whereas viruses being non cellular particles are not classified this way. Alternatively, scientists have developed variety of methods to characterize this vast virosphere. Which include more and more on genetic characteristics, observable traits, also evolutionary backgrounds (Siddell et al., 2020). Viral genomes can be organized in the form of RNA or DNA. DNA can be double stranded, single stranded (positive sense or negative sense), segmented or not. Virologists classify viruses on the basis of genome sequences, host range and cycle characteristics (Chaitanya & Chaitanya, 2019). The most thorough method of classifying viruses into families and genera is offered by International Committee on Taxonomy of Viruses (ICTV). It designates viruses to families or genera on the basis of different attributes viruses share. To cope up with the increasing metagenomic data and the increase in genetic diversity in the virosphere, ICTV has introduced a taxonomic system similar to the Linnaean system. Viral taxonomy has undergone expansion from 5 to 15 ranks in hierarchy, which better encompasses and categorize complex virus ecosystems (Gibbs, 2020).

1.1.3 Viral life cycle

The virosphere showcase wide range of specific molecular interactions between the host cell and the virus. Diversity can be seen reflected in variation throughout the life cycle of a typical virus (Rothenburg & Brennan, 2020). A virus can infect different ‘host ranges’, observing the range we can detect what kind of cells it can infect successfully. The life cycle of a virus generally comprises three primary stages, which involve entering the host cell, replicating the genome, and assembling the virion. Lytic viruses burst the host cell and after replication,

whereas lysogenic viruses insert their genome into the host cell causing chronic infection. Studying the viral capsid is vital in understanding the process of entering the host cell as it serves as a protective coat for the virus, shielding its genetic material while taking control of essential cellular machinery (Ryu, 2017).

1.2 Viruses of microbes

The field of microbiology dedicated to the study of viruses that infect microorganisms is both fascinating and complex. These viruses, known as phages, mycoviruses, and giant viruses, have a significant impact on the behavior of microbial communities and ecosystems. They can infect bacteria, fungi, and protists, which affects their numbers, genetic variation, and roles in the ecosystem. By studying these distinct classes of microbial viruses, we can gain unique insights into the intricate interactions between viruses and their hosts, and understand their roles in microbial ecology, evolution, and biotechnology applications (Hyman & Abedon, 2012).

1.2.1 Phages

Phages, which are also known as bacteriophages, are viruses that specifically target and reproduce within bacteria. They play a crucial role in controlling bacterial populations in various environments, as they infect and eventually destroy their hosts. Phages have been extensively researched for their potential applications in biotechnology, such as phage therapy to combat bacterial infections, and their influence on microbial communities and evolution of bacteria (Clokie et al., 2011).

1.2.2 Mycoviruses

Mycoviruses are a special type of virus that infects fungi, such as molds, yeasts, and other fungal microorganisms. These viruses have diverse genetic characteristics that can lead to both positive and negative effects on their fungal hosts. While some mycoviruses are considered as potential biological control agents for fungal diseases, others can significantly impact the virulence and behavior of fungi in different agricultural and environmental settings. Therefore, it is crucial to continue researching the fascinating world of mycoviruses and their interactions with fungi (Hough et al., 2023).

1.3 Giant Viruses

1.3.1 The term "Giant virus"

The fascinating journey of giant viruses began with the identification of first giant virus in 2003. Later on, this led to discovery of many other giant viruses, including the description of novel viral groups.

Continues findings of these giant viruses has led to the emergence of new viral families that we now acknowledge as phylogenetic superfamily nucleocytoplasmic large DNA viruses (NCLDV). NCLDV consist of many viral families from different orders and include unclassified viruses sharing same characteristics. Most notable features being relatively large size, large genome size, and sharing common genes (Iyer et al., 2006).

Regardless of above mentioned features, defining what constitutes a giant virus is still somewhat arbitrary. Researchers and different reviewers have suggested a varying categorizing criterion for giant viruses. Some researchers prefer simple factors, like minimum genome size of 200kb (a third of bacteria's genome size) and particle sizes larger than 200nm. Others attempt to characterize on the basis of similar features including similarities in genes, morphology, and replication cycle. Whereas theories regarding evolutionary nature of these unique viruses is still diverse and elusive (Wilhelm et al., 2017).

Although ICTV does not officially recognized the NCLDV superfamily, initially it has included five viral families. Poxviridae, is one of them which was once considered to contain the largest known viruses i.e., poxvirus. Many small NCLDV viruses are not considered giants, as the term 'giant virus' was used much earlier in the context of some algae infecting NCLDV. In retrospect, many viruses characterized until 1970s are in fact potential giant viruses. Unfortunately, regarding its ambiguity and subjectivity, the term giant virus cannot be included with the term NCLDV and is often used to describe protist-infecting viruses (Mönttinen et al., 2021).

In the past 20 years, interest in the field of giant viruses and increased significantly. Resulting in addition of new virus families to the NCLDV superfamily i.e., *Mimiviridae*, *Marseilleviridae,* and the proposal of several others like *pandoraviridae.* Furthermore, there have been claims that there are in reality more recognized giant viruses like *Faustoviruses*, and other like *Pithovirus* and *Mollivirus* which only have one or just few members. Contemplating, continuous addition of giant viruses, and the uncertain nature of viral taxonomy itself, the above

mentioned classification is probable to change. One of the types of research suggests putting all the giant viruses into a new order called, *Megavirales (Colson et al., 2013)*.

The delay of almost a century in the discovery of giant viruses was due to the epistemological notion that viruses are particles that are smaller than 0.2 µm. **Figure 1** shows how the 0.2 µm barrier not just stops giant viruses but also small microorganisms. Lwoff's criteria of distinguishing viruses from cells did not incorporate size as a standard, however it was main criteria which separated viruses from microbes. Giant viruses break the limits defined by Lwoff's criteria, and reasons are not just limited to particle size but also to genomic content.

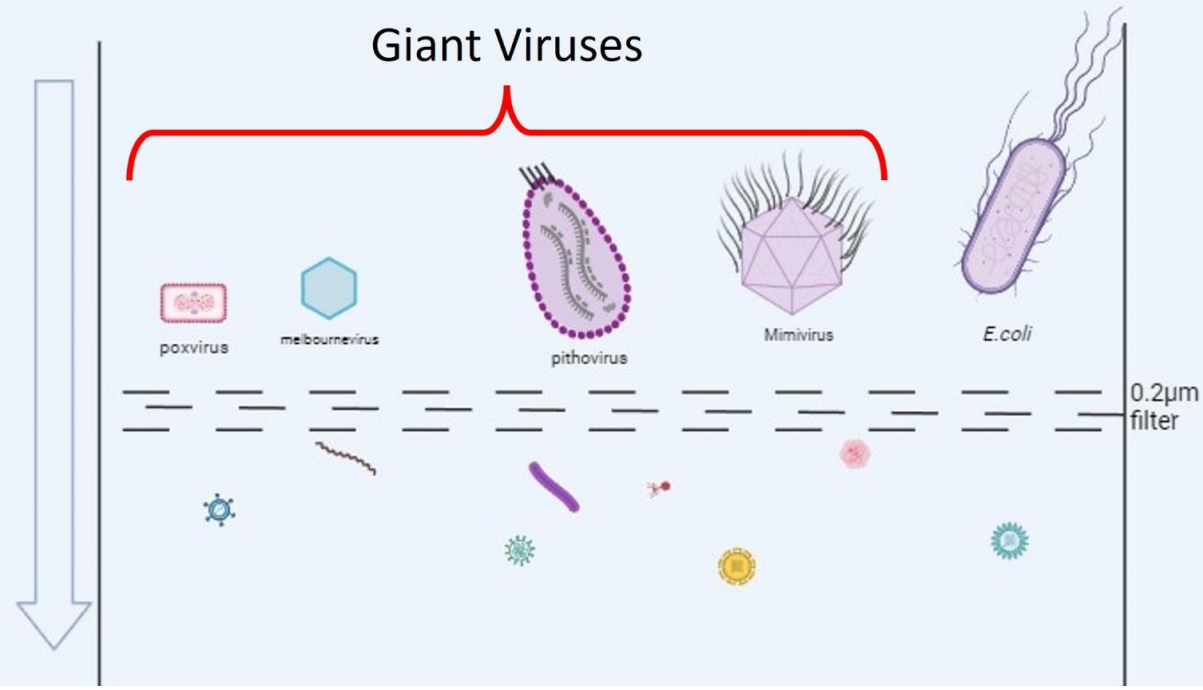


Figure 1: *An illustration of how 0.2 µm filter traps different microbes while filter out smaller particles. We can see that E. coli (a bacteria) and giant viruses like mimivirus and Pithovirus can get trapped in the filter, representing the epistemological barriers. Image created with BioRender.com, (2023)*

1.3.2 Genome, proteome, and morphology

The study of proteomes in giant viruses can be considered a highly intricate and demanding task. Notably, a significant proportion of the giant virus genome is composed of ORFans, which are open reading frames lacking homologues in previously registered genomes. The prevalence of these uncharacterized proteins can be remarkably high, ranging from 50% to over 80%, which is substantially greater than the percentages observed in bacterial proteomes (30-40%) and even in humans (around 60%). Nevertheless, it is encouraging to see that, as more giant virus families are discovered and isolated, the occurrence of ORFans is gradually diminishing. This suggests that our comprehension of these viruses and their proteins is developing, and we

are gaining a better understanding of their biological significance and evolutionary history (Brahim Belhaouari et al., 2022).

It's worth noting that viral genomes mostly consist of proteins that function either as structural elements like capsids, or non-structural, regulatory, and accessory proteins that aid in viral replication and assembly (Shammakhi, 2020). There is a significant diversity of viral gene content, with around 70% of viruses carrying fewer than 10 genes, while only 10% have over a hundred genes, and a mere 0.3% possess more than 500 genes (Hatfull, 2008). Giant viruses, on the other hand, have larger genomes, ranging from 200 kilobases to 2.5 megabases, and their predicted proteins showcase significant genome complexity (Schulz et al., 2022). It's fascinating to note that certain *Mimiviridae* family members possess genes that are linked to DNA repair, transcription machinery, translation, and even mechanisms that provide defense against virophages (Suzan-Monti et al., 2006). The intricate nature of these viruses poses a challenge to the traditional definitions of viruses. It also creates a vagueness in distinguishing between microbes and viruses, signifying that the differentiation between the two may be more of a gradient than a distinct binary division.

It has been observed that there is a general trend in which virus particle size tends to increase with the size of the virus genome, although Pithovirus appears to defy this trend (Edwards et al., 2021). Additionally, giant viruses are known to exhibit a wide range of morphologies, with most NCLDVs possessing an icosahedral or roughly icosahedral capsid structure, while others have ovoid or spherical shapes. The application of cryo-electron microscopy has led to the discovery that many icosahedral NCLDVs have similar structural characteristics. The outer capsids of these viruses are composed of protein capsomers that are arranged and organized in a comparable manner. Additionally, these viruses have an internal membrane that encloses the nucleocapsid (Fang et al., 2019). The attachment of viruses to their host is facilitated by the presence of glycoprotein-adorned fibrils on the external capsid, which varies between viruses and is a crucial characteristic (Rodrigues et al., 2015). It is believed that the presence of fibrils, combined with the larger particle size, facilitates the infiltration of giant viruses into amoebas, which have a highly selective phagocytic process (Wilhelm et al., 2017).

1.3.3 Variety of Giant virus in environment and their hosts

There is compelling evidence from recent metagenomic data indicating that NCLDV have vast host ranges and proteomic diversity and are widely present in various environmental niches. To isolate these giant viruses, researchers rely on environmental samples collected from different

locations using a co-culturing approach with amoeba. This co-culturing method has evolved over time, leading to the proliferation of what is now known as "giant viruses of the amoeba" (Colson et al., 2017).

Acanthamoeba, a free-living amoeba, is commonly used as a host in these isolation methods. *Acanthamoeba* is present in many environments and can switch between trophozoite and cyst forms depending on environmental conditions. The trophozoite form has finger-like projections called acanthopodia that aid in adhesion to surfaces, movement, and feeding. Studies have shown that *Acanthamoeba* selectively ingests individual particles larger than 0.557 µm, while smaller particles require surface accumulation before ingestion (Weisman, 1976).

Although amoeba is likely the natural hosts for some giant viruses such as *Mimivirus*, different host ranges are exhibited by most of these viruses, and their natural hosts have not yet been identified. Some studies indicate that certain NCLDVs may use algae as a host, where the virus attaches to the cell wall through adsorption. Giant viruses have been found to potentially have a crucial impact on wider natural cycles in oceanic and other ecosystems. This is especially true since they have been discovered in various unicellular protists as well as zooplankton. Additionally, there is literature suggesting the presence of *Marseilleviruses* in human samples, although these findings remain subject to ongoing debate and scrutiny of the pathogenic role these viruses may present (Takemura, 2016).

1.3.4 Life cycle

1.3.4.1 Entry Mechanism

Recent studies have shown that giant viruses have a diverse range of methods for entering host cells, unlike typical viruses. They utilize several host cell surface molecules to initiate infection, making them more effective at infecting various organisms. Once attached to the host cell, these viruses can enter through two main pathways: endocytosis and membrane fusion. The process of endocytosis involves the uptake of virus particles into host cell vesicles, creating an endosome. On the other hand, membrane fusion allows the virus to fuse directly with the host cell's membrane, releasing the viral genome into the cytoplasm of the host (Sobhy, 2017).

1.3.4.2 Genome Replication and Transcription

One of the unique characteristics of giant viruses is their ability to create specialized compartments within the host cell called replication factories. These factories provide an ideal environment for the virus to replicate and transcribe its genetic material. Recent studies have

identified various factors, both from the host and the virus, that play a role in the formation and maintenance of these factories. Additionally, giant viruses have evolved sophisticated strategies to manipulate host cellular processes and evade the host immune response. Novel research has shed light on how viral proteins can interfere with host signaling pathways, impair the host's antiviral defense mechanisms, and exploit host resources to promote their own replication (Moniruzzaman et al., 2023).

1.3.4.3 Unique Aspects of Giant Virus Replication

The study of *Mimivirus*, a well-studied giant virus, has provided valuable insights into the complex replication processes of these viruses. The latest academic studies on *Mimivirus* have revealed the importance of dedicated viral factories and the synchronization of multiple viral procedures throughout the replication process. Moreover, the identification of various viral proteins and enzymes, such as DNA and RNA polymerases, and helicases, has highlighted their critical roles in the replication of giant viruses. Thus, comprehending the functions and interplay of these proteins is essential for unraveling the intricate replication mechanisms of these viruses (Rolland et al., 2021). **Figure 2** presents a proposed depiction of the infection cycle of a giant virus.

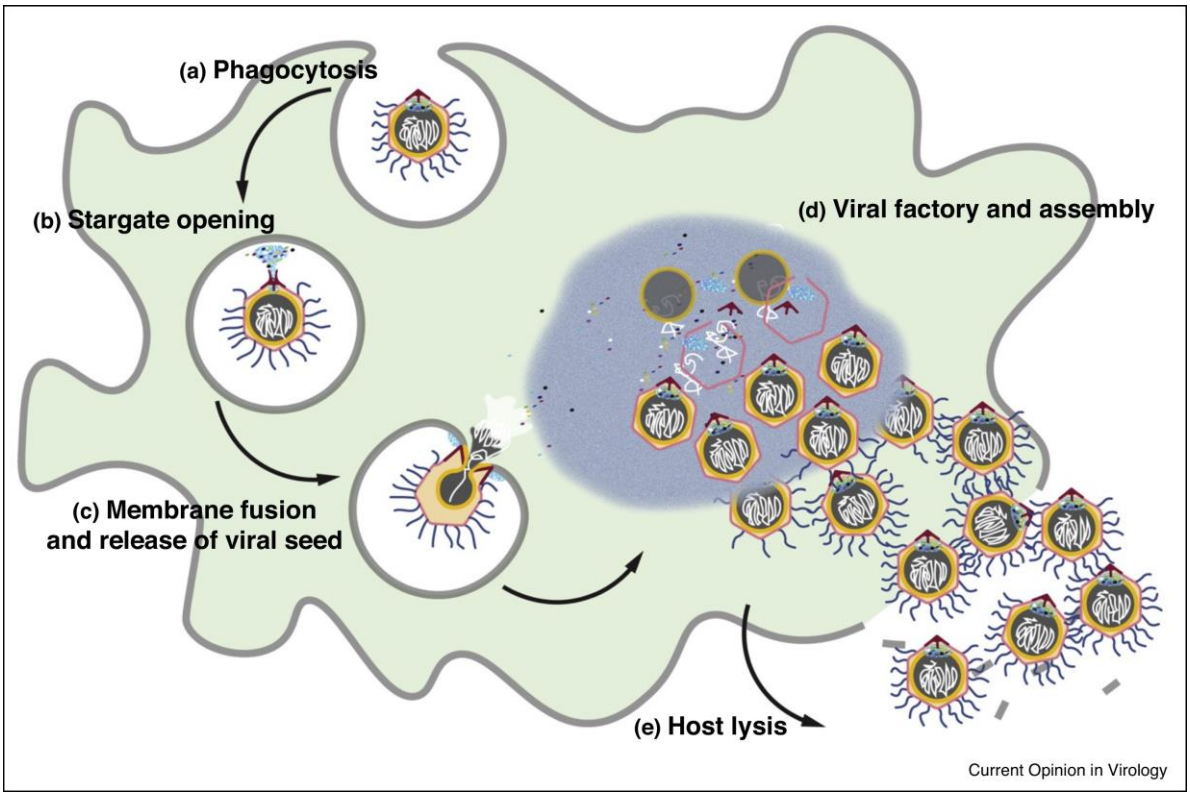


Figure 2: *Proposed illustration of Samba virus life cycle in Acanthamoeba castellanii (dos Santos Oliveira et al., 2021)*

2 Aim and Objectives

Although a lot has been described in regard to viruses of unicellular eukaryotes, there are still large gaps in this field regarding biases created by pure genomic work and by isolation efforts using only reference hosts (as in the case of *Acanthamoeba* and giant viruses). The current research thesis aims to fill up the knowledge gaps present in the field of viruses of unicellular eukaryotes exploration. The focus of this research is to get the knowledge of novel unicellular eukaryotes and the viruses infecting them.

The main objective is to discover new viruses from the Arctic region. It will be achieved by the following sub-objectives: isolation of arctic unicellular eukaryotes to use as local hosts, characterization of the isolated hosts and isolation of viruses. Additionally, secondary objective is to isolate viruses using reference strains of *Acanthamoeba*, common hosts used for giant viruses research.

3 Materials and Methods

3.1 Materials

3.1.1 Biological and environmental samples

Samples for the project were divided into two parts. One for the unicellular eukaryotic host isolation and one for the virus isolation as an infectious agent.

3.1.1.1 Samples used for unicellular eukaryote isolation

Environmental samples collected during the BIO-3612 course cruise in 2022 were used for amoeba isolation on non-nutrient agar (NNA) plates. Additionally, samples provided by the aquaculture and environmental group (https://en.uit.no/forskning/forskningsgrupper/gruppe?p_document_id=515427) were used for isolations in tissue culture flasks. This group focuses on the fish-environment interaction. This group made primary culture of salmon scales and they observed contamination in two cultures. They tested for bacterial contamination which resulted in that these are not bacteria and hypothesized that the contaminant could had been a protist.

3.1.1.2 Samples used for virus isolation.

For this project several samples were collected from different locations of the arctic region and the city of Tromsø. Details of the sample are given in **Table 1**. The samples were stored in 1.5ml Eppendorf tubes. Antibiotic mixture was added, consisting of Amphotericin B (0.25 µg/ml), Ciprofloxacin (0.004 mg/ml), and Vancomycin (0.004 mg/ml). The samples were stored in a -20C freezer before usage. Sample filtration was not performed as our main focus was giant viruses. Usage of antibiotics was done to prevent any bacterial or fugal contamination in the samples accompanied with freeze-thawing at least once to disrupt most of the cellular life on them. Antibiotics were also supplemented in the culture media used for viral isolation to prevent bacterial growth.

Table 1: *List of samples used for virus isolation.*

Sample #	Collection Date	Location	Information
1	7.3.2022	UiT-NFH tanks	Mixed sea animals
2	7.3.2022	UiT-NFH tanks	Mostly shrimps
3	7.3.2022	UiT-NFH tanks	Only sea urchins
4	22.3.2022	Havbruksstasjonen	Arctic charr from Hammerfest
5	22.3.2022	Havbruksstasjonen	Arctic charr (smaller) from Dvergrøye (midged), born in 2020
6	22.3.2022	Havbruksstasjonen	Arctic charr (Svalbard)
7	22.3.2022	Havbruksstasjonen	Arctic charr from Hammerfest born in 2020
8	22.3.2022	Havbruksstasjonen	Arctic charr from Hammerfest born in 2019
9	22.3.2022	Havbruksstasjonen	Arctic charr from Hammerfest born in 2019
10	22.3.2022	Havbruksstasjonen	Crabs (sea water)
11	22.3.2022	Havbruksstasjonen	Crabs (sea water)

12	22.3.2022	Havbruksstasjonen	Few dead shrimp (sea water)
13	22.3.2022	Havbruksstasjonen	Few dead shrimp (sea water)
14	22.3.2022	Havbruksstasjonen	Snowcrabs
15	22.3.2022	Havbruksstasjonen	Salmon (sea water)
16	22.3.2022	Havbruksstasjonen	Salmon eggs, in dark cold fresh water
17	22.3.2022	Havbruksstasjonen	Salmon eggs, in dark cold fresh water
18	22.3.2022	Havbruksstasjonen	Salmon eggs, in dark cold fresh water
19	22.3.2022	Havbruksstasjonen	Salmon eggs, in dark cold fresh water
20	22.3.2022	Havbruksstasjonen	Cod
21	22.3.2022	Havbruksstasjonen	Cod
22	22.3.2022	Havbruksstasjonen	Salmon
23	22.3.2022	Havbruksstasjonen	Salmon (sea water)
24	22.3.2022	Havbruksstasjonen	Lumpfish (small)
25	22.3.2022	Havbruksstasjonen	Lumpfish (large)
26	22.3.2022	Havbruksstasjonen	Lumpfish (large)
27	22.3.2022	Havbruksstasjonen	Combined outlet water from salmon
28	06.4.2022	UiT-NFH sea water (5th floor)	Sea water from the tank on the 5th floor.
29	06.4.2022	UiT-NFH sea water (5th floor)	Sea water from the tank on the 5th floor.
30	06.4.2022	UiT-NFH sea water (5th floor)	Sea water from the tank on the 5th floor.
31	08.4.2022	Borgtun ground water mine	Underground water leaking from the street - melting snow
32	10.4.2022	Winter trail Tromsø	Collected in a stream from a frozen lake near UiT
33	10.4.2022	UiT "river"	Collected near the drain in front of the BFE
34	10.4.2022	UiT "labyrint" fountain	Collected near the drain
35	10.4.2022	Rock/bryophyte/lichen water	Water draining through all from melting ice, collected near UiT
36	13.4.2022	Bathavna, upstream	River water
37	13.4.2022	Bathavna, downstream	Below bridge, strong current
38	13.4.2022	Bathavna, bird droppings	Near sea, mixed with water / 38+39 mixed after filtration
39	13.4.2022	Bathavna, bird droppings	Near sea, mixed with water / 38+39 mixed after filtration
40	14.4.2022	Stakkevollvegen 29	Sea, low tide
41	20.4.2022	69.67519642603253, 18.978714535183595	Collected from the inlet sewage (avoiding solid material)
42	20.4.2022	69.67519642603253, 18.978714535183595	Collected from the inlet sewage (avoiding solid material)
43	20.4.2022	69.67519642603253, 18.978714535183595	Collected from the inlet sewage (avoiding solid material)
44	20.4.2022	69.67519642603253, 18.978714535183595	Collected after filtration
45	20.4.2022	69.67519642603253, 18.978714535183595	Collected after filtration
46	20.4.2022	69.67519642603253, 18.978714535183595	Collected after filtration
47	4.2022	Water boiler in Håkøya	Collected on different days around Easter 2022
48	4.2022	Water boiler in Håkøya	Collected on different days around Easter 2022
49	4.2022	Water boiler in Håkøya	Collected on different days around Easter 2022
50	4.2022	Water boiler in Håkøya	Collected on different days around Easter 2022
51	4.2022	Water boiler in Håkøya	Collected on different days around Easter 2022
52	4.2022	Water boiler in Håkøya	Collected on different days around Easter 2022
53	8.9.2022	BIO3612 cruise	Sea water (surface)
54	8.9.2022	BIO3612 cruise	Sea water (surface)
55	8.9.2022	BIO3612 cruise	Sea water from collected sediments (bucket 1)
56	8.9.2022	BIO3612 cruise	Sea water from collected sediments (bucket 2)
57	8.9.2022	BIO3612 cruise	Sea water from animal tray (post students processing - long incubation)
58	8.9.2022	BIO3612 cruise	Sea water from collected sediments (bucket 3, Klara trip)
59	N/A	deep sea vents	Provided by the DeepSeaQuence project (University of Bergen)
60	N/A	deep sea vents	Provided by the DeepSeaQuence project (University of Bergen)
61	N/A	deep sea vents	Provided by the DeepSeaQuence project (University of Bergen)

62	N/A	deep sea vents	Provided by the DeepSeaQuence project (University of Bergen)
63	N/A	deep sea vents	Provided by the DeepSeaQuence project (University of Bergen)
64	N/A	deep sea vents	Provided by the DeepSeaQuence project (University of Bergen)
65	N/A	deep sea vents	Provided by the DeepSeaQuence project (University of Bergen)
66	N/A	deep sea vents	Provided by the DeepSeaQuence project (University of Bergen)
67	N/A	deep sea vents	Provided by the DeepSeaQuence project (University of Bergen)
68	N/A	deep sea vents	Provided by the DeepSeaQuence project (University of Bergen)
69	N/A	deep sea vents	Provided by the DeepSeaQuence project (University of Bergen)
70	N/A	deep sea vents	Provided by the DeepSeaQuence project (University of Bergen)
71	N/A	deep sea vents	Provided by the DeepSeaQuence project (University of Bergen)
72	N/A	deep sea vents	Provided by the DeepSeaQuence project (University of Bergen)
73	N/A	deep sea vents	Provided by the DeepSeaQuence project (University of Bergen)
74	N/A	deep sea vents	Provided by the DeepSeaQuence project (University of Bergen)
75	N/A	deep sea vents	Provided by the DeepSeaQuence project (University of Bergen)
76	N/A	deep sea vents	Provided by the DeepSeaQuence project (University of Bergen)
77	N/A	deep sea vents	Provided by the DeepSeaQuence project (University of Bergen)
78	N/A	deep sea vents	Provided by the DeepSeaQuence project (University of Bergen)
79	N/A	deep sea vents	Provided by the DeepSeaQuence project (University of Bergen)
80	24.9.2022	Stakkevollvegen 29	sample from the seashore, near macroalgae. Low tide
81	24.9.2022	Stakkevollvegen 29	sample from the seashore, near macroalgae. Low tide
82	24.9.2022	Stakkevollvegen 29	sample from the seashore, near macroalgae. Low tide
83	25.9.2022	Bathavna, high tide	before bridge (sea side)
84	25.9.2022	Bathavna, high tide	after bridge (river side)
85	25.9.2022	Bathavna, high tide	after bridge (river)
86	10.3.2023	Salmon scales from the aquaculture station	Scales collected by Divia, kept at 4°C with antibiotics for 10 days

3.1.2 MY75S media

The media was prepared according to the concentrations mentioned in **Table 2**.

Table 2: *List of components for the preparation of MY75S media*

Components	quantity/liter
Natural seawater, Filtered	750ml
Deionized water	250ml
Malt extract	0.1g
Yeast extract	0.1g

Added the components in 1 liter distilled water and sterilized by autoclavation at 121°C, for 15 minutes at 15 PSI.

3.1.3 PYG (Peptone Yeast Extract Glucose Broth) Media

The media was prepared according to the concentrations mentioned in **Table 3**.

Table 3: *List of components for the preparation PYG media(Thomas et al., 2006).*

Components	quantity/liter
NaCl	120 mg
$MgCl_2.6H_2O$	3 mg
Na_2HPO_4	142 mg
Kh_2PO_4	136 mg
$CaCl_2$	3 mg
$FeSO_4$	3 mg
Peptone	20 g
Yeast extract	20 g
Glucose	18 g

Added these components in 1 liter of distilled water and sterilized by autoclavation.at 121°C, for 15 minutes at 15 PSI.

3.1.4 Page's modified Neff's amoeba saline (PAS) buffer

Following **Table 4** shows the composition of PAS buffer.

Table 4: *List of components in the PAS buffer(Thomas et al., 2006).*

Components	quantity/liter
NaCl	120 mg
$MgSO_4.7H_2O$	4 mg
$CaCl_2.2H_2O$	4 mg
Na_2HPO_4	142 mg
KH2PO4	136 mg

Added these components in 1 liter of distilled water. Sterilized by autoclavation.at 121°C, for 15 minutes at 15 PSI.

3.1.5 Chemical reagent, kits, and buffers

In this study, the DNeasy Blood and Tissue kit (manufactured by QIAGEN, Valencia, CA, USA), a commercially available product, was employed for the process of DNA extraction. Enzymatic pretreatment with proteinase K was utilized to enhance cell lysis. DreamTaq Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) was used for PCR amplifications. The final step involved purification of the resulting amplicons using the QIAquick PCR Purification kit (also manufactured by QIAGEN, Valencia, CA, USA). Instructions from the manufacturers were followed for each of the kits used.

3.1.6 Primers, PCR reactions

To amplify the 18S DNA region from our samples we use GGF and GGR primers and reagents as shown in the **Table 5** and **Table 6**.

Table 5: *PCR primers used.* (Bellemain et al., 2010; Dmitry G. Zagumyonnyi et al., 2021)

Primers	Sequence
intF	5′-GTTTCAGCCTTGCGACCATACT-3′
ITS4r	5'-TCCTCCGCTTATTGATATGC-3′
ITS5f	5'-GGAAGTAAAAGTCGTAACAAGG-3'
GGF	5′-CTTCGGTCATAGATTAAGCCATGC-3′
GGR	5′-CCTTGTTACGACTTCTCCTTCCTC-3′
intR	5′-GGTAATTCCAGCTCCAATAGCGTA-3′

Table 6: *List of components for 18S PCR*

Components	1 Reaction (20µl)	4 Reactions (80µl)
Water	7	28
DreamTaq green MM	10	40
GGF primer 10µm	1	4
GGR primer 10µm	1	4
Template (DNA)	~10ng	~10ng

ITS amplification was made by using reagents mentioned in **Table 7**.

Table 7: *List of components for ITS sequencing*

Components	1 Reaction (20µl)	3 Reactions (60µl)
Water	7	21
DreamTaq green MM	10	30
ITS4r primer 10µm	1	3
ITS5f primer 10µm	1	3
Template (DNA)	~10ng	~10ng

3.1.7 Sanger sequencing

List of reaction mix for the sanger sequencing can be found in **Table 8** while primers are mentioned in **Table 5**.

Table 8: *List of components for sanger sequencing reactions.*

Components	1 reaction (µl)	13 Reactions (µl)
Big Dye	2	26
Big Dye buffer	3	39
Primer	1	Add individually
Water + DNA template	14	8 µl DNA + 174µl water = 182 µl

3.1.8 Project workflow

The workflow and the methods used in the project are depicted in **Figure 3**.

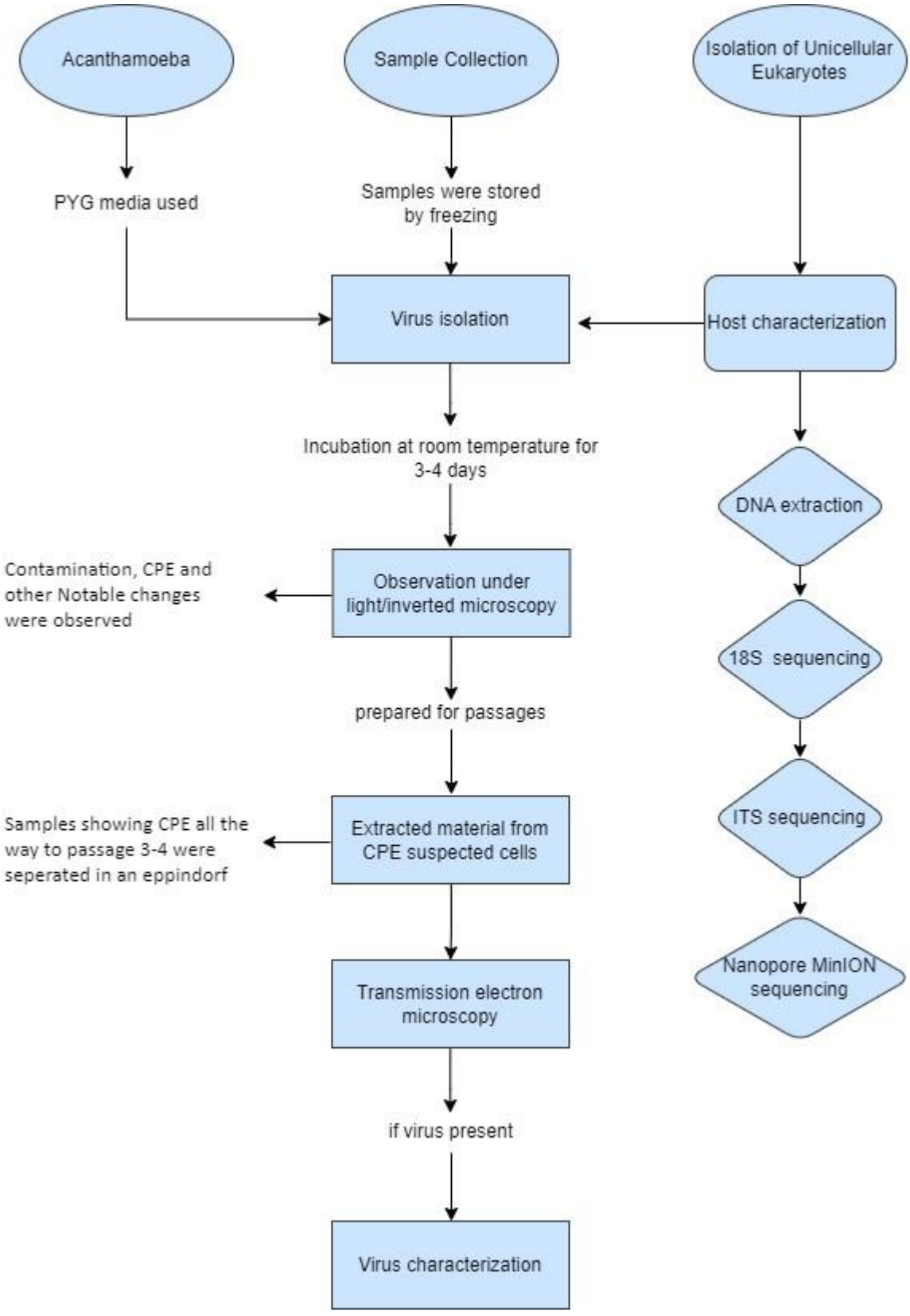


Figure 3: Overview of the workflow of project.

3.2 Methodology

3.2.1 Isolation of unicellular eukaryotes

Two approaches were used for the isolation of unicellular eukaryotes. One consisted in selecting from motile ameboid cells in solid media, whereas the second focused on isolation cells using liquid media in static cultures. Thus, samples meant for isolation were tested either in NNA agar plates (selection of arctic amoeba) or in tissue culture flasks (selection of unicellular eukaryotes from fish cell cultures).

3.2.1.1 Isolation of the arctic amoeba

To grow and isolate the arctic amoeba from environmental samples, NNA plates were prepared by being covered with dead *Escherichia coli* as a nutrient source. The *E. coli* cells were prepared by autoclaving one overnight culture, then 100 microliters were spread over the plates. After drying the environmental samples were added as drops and the plates were followed daily. Whenever growth was seen, agar blocks were cut and moved to fresh NNA plates also containing dead *E. coli* cells. The objective was to select for cells with motility, capable of grazing the dead *E. coli* in the agar plate.

3.2.1.2 Isolation of unicellular eukaryotes

As alternative to the isolation in plates, we conducted an experiment to isolate and cultivate unicellular eukaryotic cells from a fish tissue sample. We obtained samples from an aquaculture and environmental group, and these samples were cultivated in different concentrations of both PYG and MY75S media to determine the ideal growth conditions in tissue culture flasks. The concentrations we used varied from pure MY75S to 100% PYG. Subsequently, we monitored them daily using an inverted microscope. Note that antibiotics were present at all times.

3.2.2 Characterizing of the isolated unicellular eukaryotes

Different approaches were utilized to characterize the isolated unicellular eukaryote including molecular and behavioral approaches.

3.2.2.1 Molecular characterization

Molecular identification was made by 18S and ITS sequencing, using extracted DNA as template for PCRs and Sanger sequencing. We employed a two-step DNA extraction procedure, beginning with the freeze-thaw method to disrupt cellular membranes. Then extracted DNA using DNeasy Blood and Tissue kit by adhering to the guidelines suggested by the manufacturerq. Nanodrop spectrophotometer was used to quantify the extracted DNA.

To amplify the 18S gene, we utilized GGF and GGR primers with DreamTaq Green PCR Master Mix from Thermo Fisher Scientific, which allowed us to amplify the target genes. Conditions for PCR cycle followed a sequence of steps starting with 95 °C for 3 min and then continuous 35 cycles of 95 °C for 30 s, 52 °C for 30 s, 72 °C for 1.5 min, and a final extension 72 °C for 5 min. (Dmitry G Zagumyonnyi et al., 2021). ITS PCR protocol cycle followed a sequence of steps starting with 95 °C for 3 min, and then continuous 35 cycles of 95 °C for 30 s, 50 °C for 30 s, 72 °C for 1 min, and a final extension 72 °C for 5 min. After each PCR i.e., 18S and ITS, success of gene amplification was verified through gel electrophoresis. PCR products were run in 0.8% agarose gels stained with gel red following the manufacturer's instructions (Bellemain et al., 2010).

Once the presence of the amplified genes was confirmed, we prepared DNA samples for Sanger sequencing by cleaning and purifying the DNA using the QIAquick PCR Purification kit by adhering to the guidelines suggested by the manufacturer. Four primers were used i.e., GGF, GGR, intF, and intR. The PCR cycle was set as: 95°C for 5 minutes; 40 cycles (10 seconds at 95°C, 10 seconds at 55°C, 4 minutes at 60°C) and final extension of 4°C. After the run, the reaction products were taken to the DNA sequencing facility at UiT for further analysis.

In addition to employing the Sanger sequencing, we also leveraged the potential of Nanopore MinION sequencing, a novel approach for targeted genetic investigations. For this purpose, we opted for the Rapid sequencing kit (Nanopore Corporation, 2020) and followed the protocol provided with the kit.

3.2.2.2 Biological Characterization

To characterize the unicellular eukaryote isolated, we used different concentration levels of MY75S media supplemented with PYG media to find the optimal growth condition for the microbe in consideration. The concentrations ranged from 10% PYG in MY75S media to 100% PYG media.

We also performed a capillary chemotaxis assay to characterize the movement of cells in the wells. For this method, we utilized a 96 well plate and prepared three groups of test solutions: MY75S media supplemented with 1% mucin, MY75S media supplemented with 50% PYG, and just MY75S media. To the first column of the plate, we added 300 µl of the conditions described above (three wells for 1% mucin, three for 50% PYG and 2 for MY75S media. To the remaining wells we added 100µl of 10% PYG. After preparing the plate a cell suspension

was obtained by resuspending a confluent culture flask, which was added to a sterile recipient. Then, using a multichannel pipet, we collected 100 µl of media (of the conditions described above, contained in the first column of the plate) inside the pipet tips and inserted them in the cell suspension for 30 minutes as shown in **Figure 4**. After 30 minutes, we took the tips out and washed it in the 1st column of the plate (clean media) by dipping the tip only to remove any cells attached to the surface, without dispensing the pipette contents. The idea is that cells attracted to the conditions inside the pipet tips would be trapped there. Then, we dispensed the material in the 2nd column without contact, directly to MY75S media with 10%PYG. We changed the pipette tips and mixed through pipetting and took 100 µl and suspended in the next column (thus making a 1/2 dilution). We kept diluting in a 1/3 factor until column 12, reaching a final dilution of 1/188098. After the dilutions we incubated the plate at room temperature and followed the growth (or not) of cells in every well. The hypothesis is that if more cells were attracted to a given condition, then we would detect cell growth in more dilutions. If no chemotaxis was seen, then all cell preparations would be found at similar dilutions. The map of the 96 well plate is given in the **Table 11**.

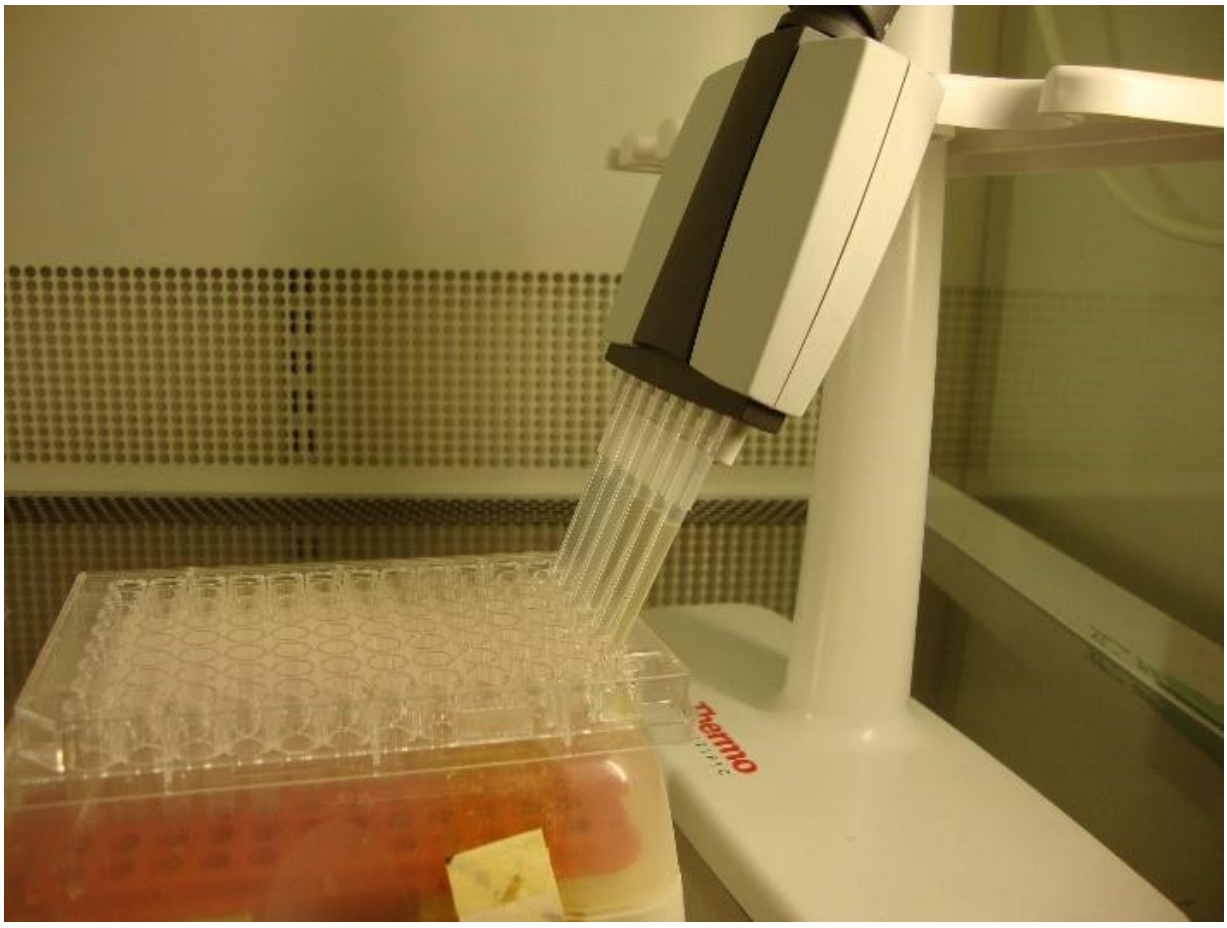


Figure 4: *Example of an adapted system to measure chemotaxis.*

3.2.3 Virus Isolation

To isolate the viruses from the samples we used two different strategies in our first approach we used *Acanthamoeba polyphaga* and *Acanthamoeba castellanii* (conventional reference hosts) to isolate the viruses, while for the second approach we used *Cystobasidium* (the unicellular eukaryote isolated in this project).

3.2.3.1 Virus isolation in Acanthamoeba

Virus isolation in Acanthamoeba was made by mixing amoeba cells with the samples to be used in 96 well plates. The final volume for each of the well was 200 µl of cell suspension including 50 µl of sample in passage 1 or of the material of the previous passages in subsequent ones. Prior to commencing the 96 well plates were seeded with cells to a final confluency of around 75%. Thirty-one samples were tested against two host species: *A. castellanii* and *A. polyphaga*. The map of the 96 well plate is shown in **Table 9**.

Table 9: *Map of 96 well plate for virus isolation. On the left side of the plate samples were screened on A. Polyphaga while right side of the plate was A. castellanii was used. C represents the control wells which were 9 in number. The numbers in the well indicate the original sample number.*

	1	2	3	4	5	6	7	8	9	10	11	12
A	**28**	**36**	**50**	**58**	**C**			**28**	**36**	**50**	**58**	**C**
B	**29**	**37**	**51**	**80**	**C**			**29**	**37**	**52**	**80**	**C**
C	**30**	**38**	**52**	**81**	**C**			**30**	**38**	**51**	**81**	**C**
D	**31**	**39**	**53**	**82**	**C**			**31**	**39**	**53**	**82**	**C**
E	**32**	**40**	**54**	**83**	**C**			**32**	**40**	**54**	**83**	**C**
F	**33**	**47**	**55**	**84**	**C**			**33**	**47**	**55**	**84**	**C**
G	**34**	**48**	**56**	**85**	**C**			**34**	**48**	**56**	**85**	**C**
H	**35**	**49**	**57**	**C**	**C**			**35**	**49**	**57**	**C**	**C**
	A. Polyphaga							*A. castellanii*				

Antibiotics (0.25µg/ml of Amphotericin B, 0.004 mg/ml of Vancomycin, and 0.004 mg/ml of ciprofloxacin) were used during the virus isolation process. For the control wells 50 µl of sterile PAS including antibiotics were added instead of samples. The culture plate was incubated at room temperature and checked every day under inverted light microscope for changes. This step was indicated as 1st passage. Changes in morphology, cytopathic effect (CPE) and bacterial and fungal contamination and cell death in comparison of control group was observed. After observation the plate was frozen in -20°C and frozen until next passage.

For the following passages same protocol was implemented but this time exposing fresh cells to the 50 µl of sample from previous passage after at least one time freeze-thawing. The passage process can be seen in **Figure 5**. After passage 3 all the sample without any effect are considered negative while the one with turbidity are contaminated by bacterial growth while clear samples with CPE are considered for potential viruses.

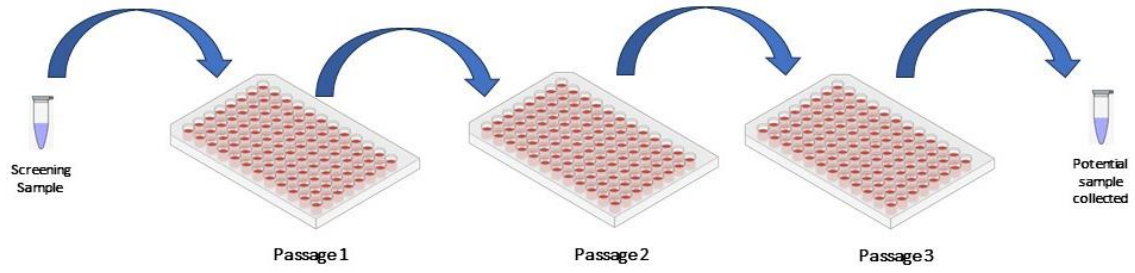


Figure 5: *Passage procedure from passage 1 to 3*

3.2.3.2 Virus isolation in *Cystobasidium*

Virus isolation in *Cystobasidium* was made by using a 96 well plate, in a similar way to what was made for Acanthamoeba. 86 samples were used as can be seen in **Table 10** along with antibiotics. Same as previous method, volume and passage strategy was implemented. One distinction was that MY75S media was used instead of PYG. The culture plate was incubated at room temperature and checked every day under inverted light microscope for changes. This step was indicated as 1st passage. Changes in morphology, cytopathic effect (CPE) and bacterial and fungal contamination and cell death in comparison of control group was observed. After observation the plate was frozen in -20°C and frozen until next passage.

Table 10: *Map of 96 well plate for virus isolation in Cystobasidium. C represents the control while numbers refer to the sample number.*

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	2	3	4	5	6	7	8	9	10	11	12
B	13	14	15	16	17	18	19	20	21	22	23	24
C	25	26	27	28	29	30	31	32	33	34	35	36
D	37	38	39	40	41	42	43	44	45	46	47	48
E	49	50	51	52	53	54	55	56	57	58	59	60
F	61	62	63	64	65	66	67	68	69	70	71	72
G	73	74	75	76	77	78	79	80	81	82	83	84
H	85	86	C	C	C	C	C	C	C	C	C	C

For the following passages same protocol was implemented but this time exposing fresh cells to the 50 µl of sample from previous passage after at least one time freeze-thawing. The passage process can be seen in **Figure 6**. At the end of passage 3 we determined which samples were negative and moved forward only with suspected samples, up to passage 5. After passage 5 all the sample without any effect are considered negative while the one with turbidity are contaminated by bacterial growth while clear samples with CPE were considered for potential viruses.

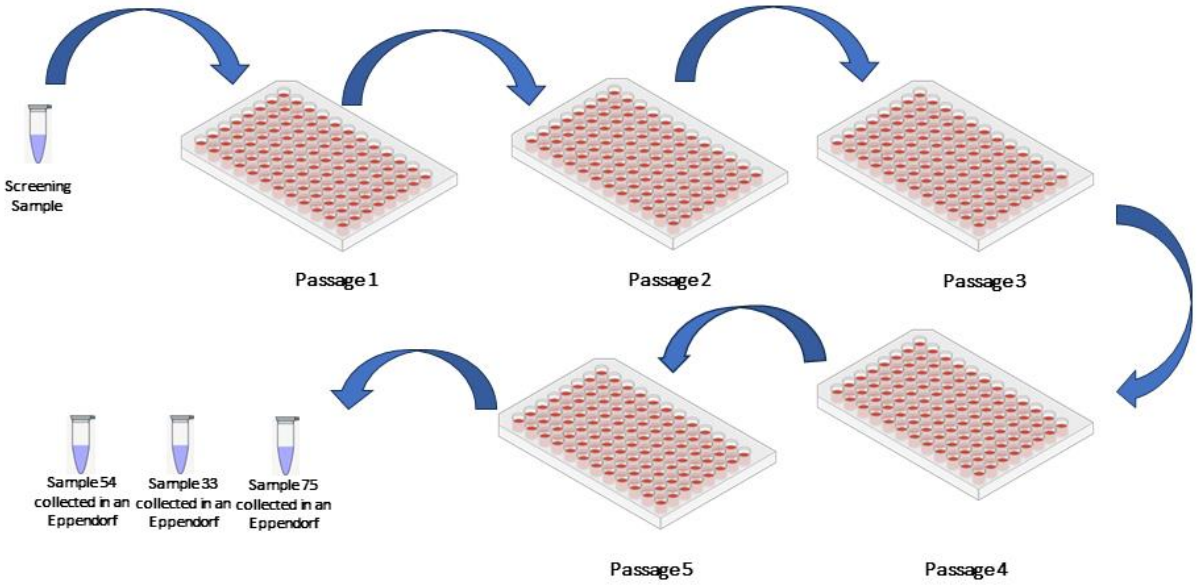


Figure 6: *Passage procedure from passage 1 to 5. Image source: https://commons.wikimedia.org/wiki/File:202012_Cell_culture_plate_size_96_wells_with_medium.svg.*

3.2.3.3 Transmission electron microscopy (TEM)

Control and infected cells were pelleted by centrifugation and the cell pellets embedded for thin sectioning. Firstly, cells, especially those growing adherently, have their culture medium removed and are immediately treated with a fixative solution. In cases of sparse cell population, cells are harvested through scraping, pelleted, and resuspended in a 12% gelatin solution, with stringent temperature control to preserve their integrity. Following fixation for a minimum of 4 hours, cells undergo multiple washes in a buffer solution to remove excess fixative. Subsequently, osmication is carried out over an extended period, with the tissue being immersed in a 1% OsO4 solution. Additional buffer washes follow to ensure optimal sample preparation. Followed by Dehydration step involving a graded series of ethanol and acetone solutions are employed for this purpose. Finally, embedding the dehydrated samples in a plastic/acetone mixture, allowing for meticulous polymerization and storage at -20°C. Thin sections were observed in a TEM microscope at the microscopy center. (Cocchiaro et al., 2008; Pokrovskaya et al., 2012).

Figure 7: *Transmission electron microscope at the MH building of the UIT.*

4 Results

4.1 Isolation of unicellular eukaryotes from the arctic region

The first step of this master project was focused on finding a local unicellular eukaryote to use as host for virus isolation. Two strategies were used to find out a unicellular host, i.e., i) isolation of amoeba cells using NNA plates covered with dead *E. coli* cells, ii) isolation of contaminants found in tissue culture samples of salmon scales.

While screening samples on NNA culture plates and utilizing dead *E. coli* as a nutrition source for the host, we encountered hints of the presence of amoeba cells. These were passaged to new agar plates cutting blocks of the previous ones, aiming at isolating only motile cells. However, after the passages we noticed that bacterial cultures took over the samples, resulting in issues with contamination. The resulting contamination can be observed in **Figure 8**. The results in **Figure 9** are the results for contamination confirmation assay. Since all samples which contained signs of amoeba presence contaminated with bacteria, we discarded these cells.

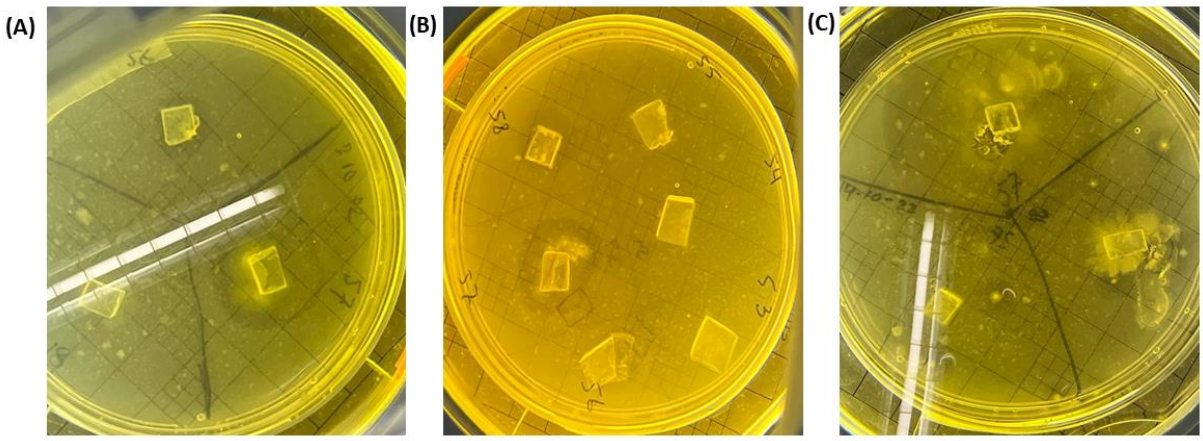


Figure 8: *NNA plates used for isolating environmental amoeba. In (A) and (B) for sample 57 we can see halo effect which can indicate amoeba growth. While in (C) we can observe contamination in the same sample which is disrupting agar in the plate.*

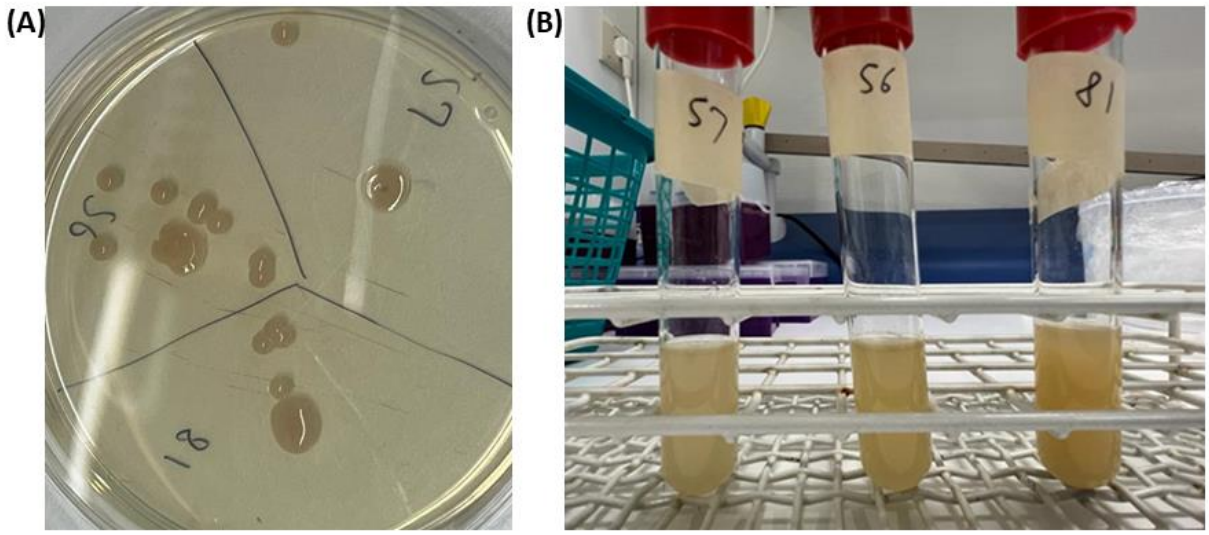


Figure 9: *Confirmation of contamination of the samples. The contamination of the culture plate was detected using growing potential suspect in plates (A) and in the test tubes (B).*

To proceed with our investigation, we obtained a sample of fish scales that appeared to have a potential unicellular eukaryote, which we considered to be an amoeba through our initial observations and description of the group who donated the samples. Although we could get it growing in static cultures, achieving the desired growth for this organism proved to be a considerable challenge as we can see in the **Figure 10**. Clusters of cells could be observed and followed for many days, but these cells often reached a point in which they stopped growing and then died. It could have been associated to the presence of fish cells, since growth often stopped after the fish cells disappeared.

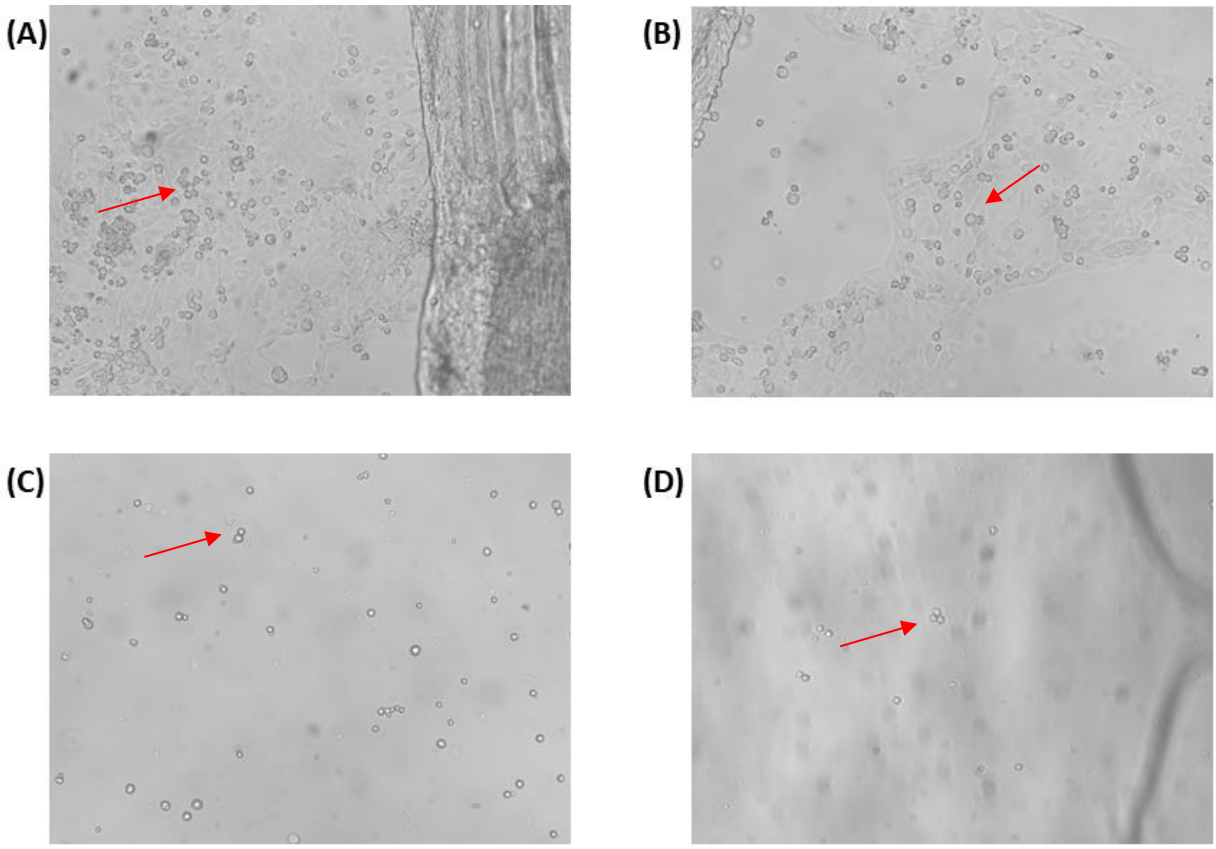


Figure 10: *Amebae-like cells isolated from a contaminated fish cell culture. (A) and (B) shows the original sample we had while (C) is the growth at day 0 while (D) is the growth at day 10. Red arrows point at Amoeba cells.*

Over the course of many weeks, we got more similar samples to test. most of the samples were negative, but one of them yielded another cell type. After days of incubation these cells grew and we manage to obtain pure cultures of them, besides enough biomass for follow up studies. The cultivated cells can be seen in **Figure 11**. These small cells had a uniform cell morphology, grew fast in tissue culture flasks, and presented a twitching motility behavior.

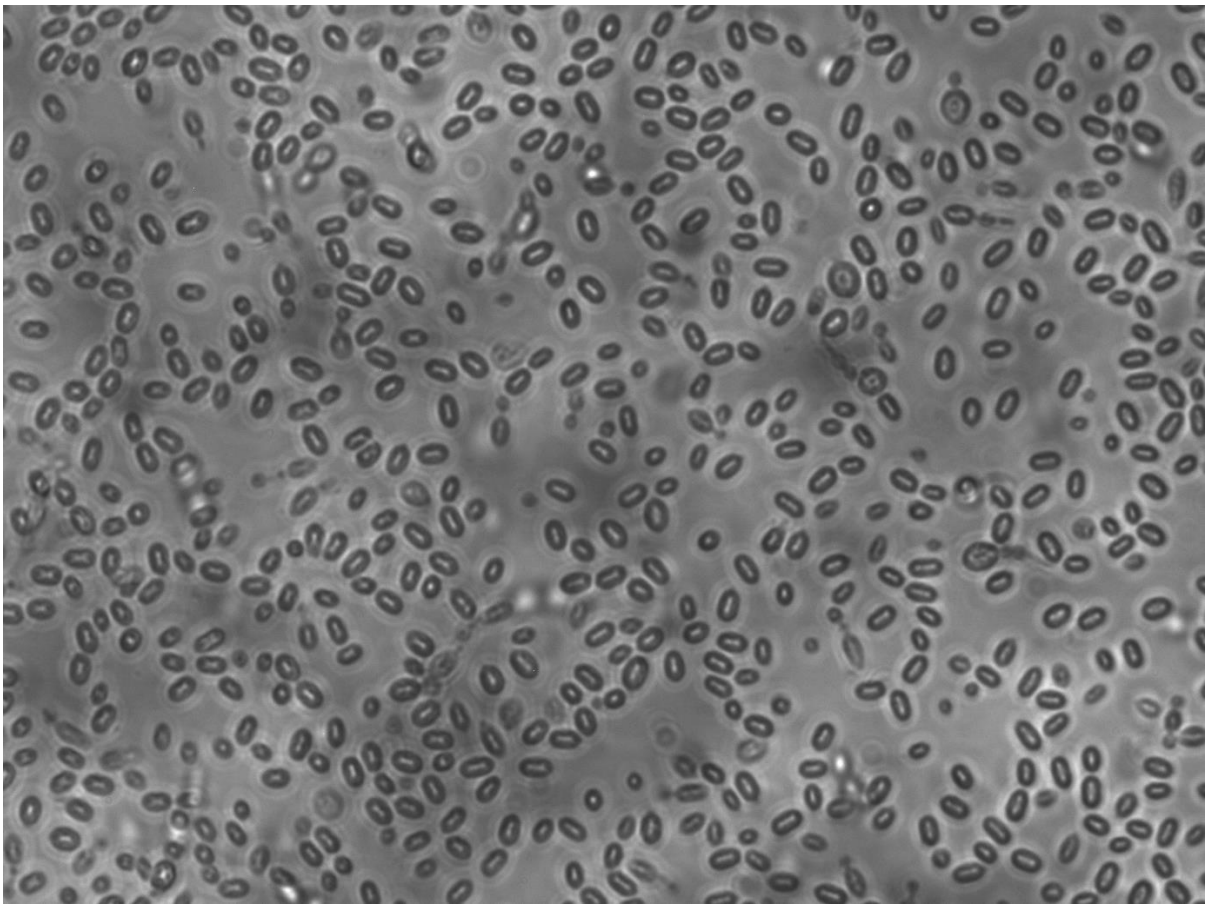


Figure 11: *Cystobasidium cells observed under 400x magnification.*

4.2 Unicellular eukaryote characterization

To initiate the characterization process of the organism found, the first step entailed the amplification of the 18S gene and ITS regions through PCR, which is then subjected to sequencing and subsequent phylogenetic analysis.

The amplified DNA band of the 18S gene was successfully obtained and visualized through gel electrophoresis, as depicted in **Figure 12**A. However, the experiment did not yield expected results for the ITS 4 and 5 region, which can be observed in **Figure 12**B.

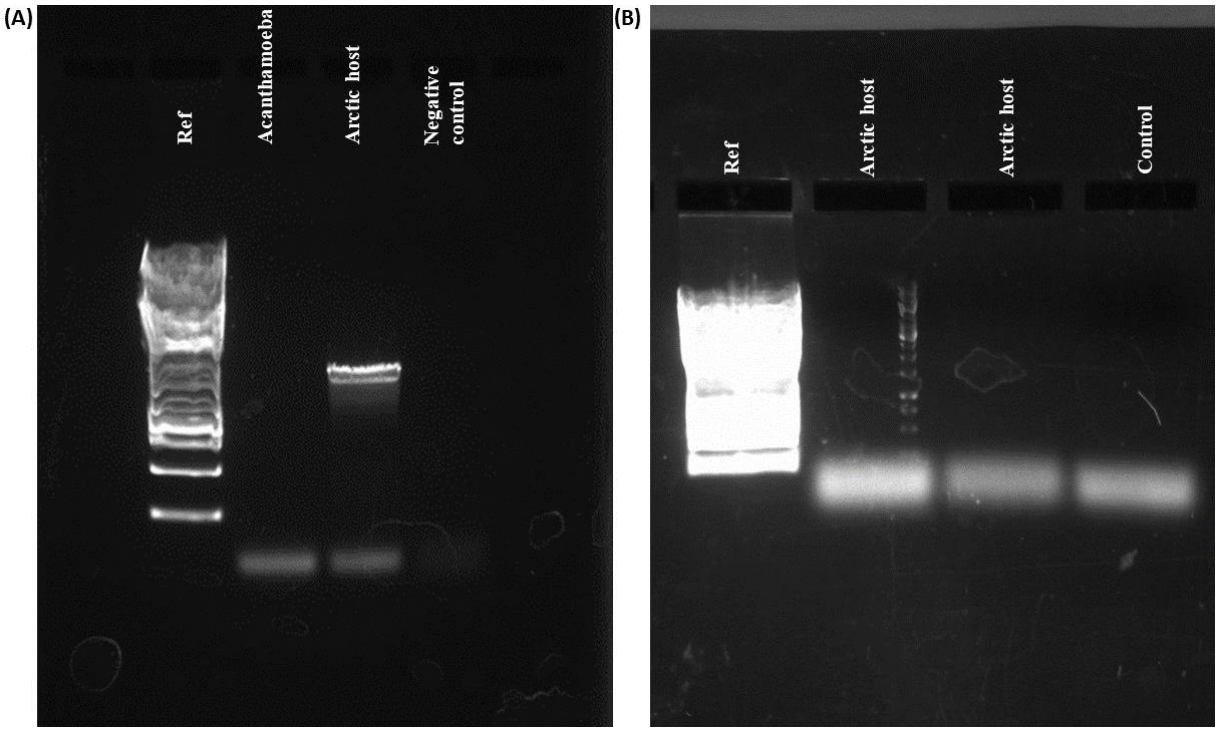
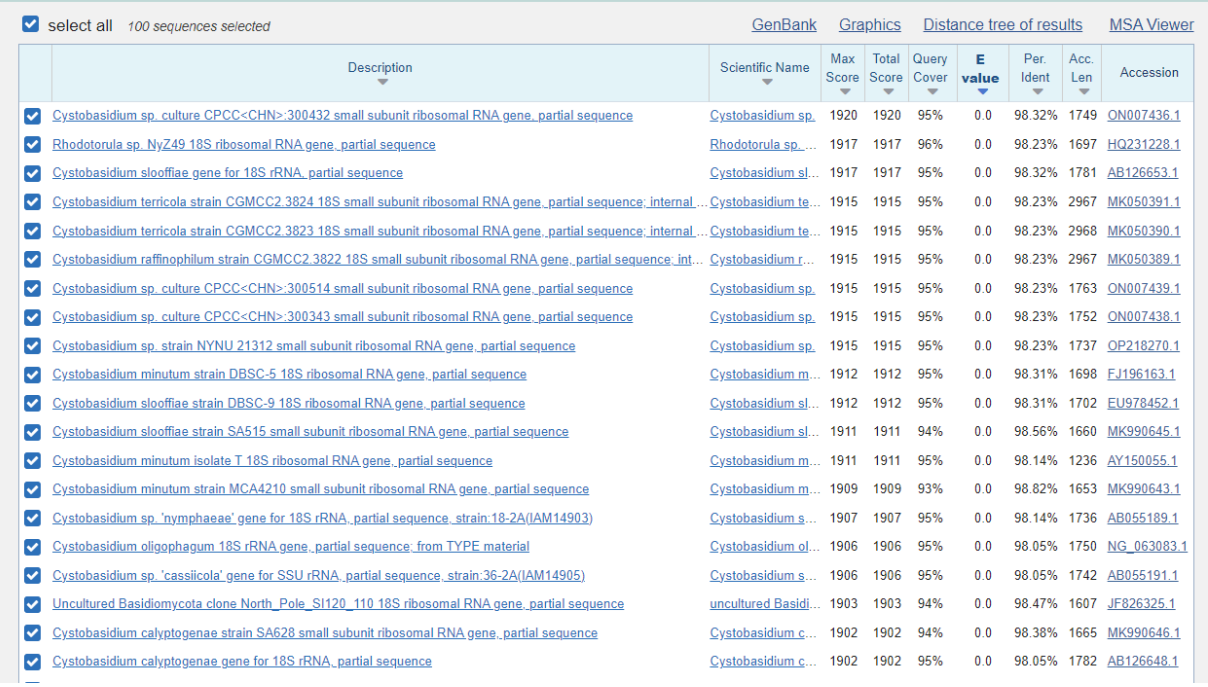


Figure 12: *Agarose gels of 18S and ITS PCR results. (A) Gel run of 18S gene PCR. (B) Gel run of ITSS 4-5 amplification PCR.*

The amplified 18S gene was subjected to Sanger sequencing, followed by a BLAST search on the National Center for Biotechnology Information (NCBI). The results, presented in **Figure 13**. revealed a higher percentage identity to *Cystobasidium* genus of yeasts in the order *Cystobasidiales*.



select all *100 sequences selected* GenBank Graphics Distance tree of results MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Cystobasidium sp. culture CPCC<CHN>:300432 small subunit ribosomal RNA gene, partial sequence	Cystobasidium sp.	1920	1920	95%	0.0	98.32%	1749	ON007436.1
Rhodotorula sp. NyZ49 18S ribosomal RNA gene, partial sequence	Rhodotorula sp. ...	1917	1917	96%	0.0	98.23%	1697	HQ231228.1
Cystobasidium slooffiae gene for 18S rRNA, partial sequence	Cystobasidium sl...	1917	1917	95%	0.0	98.32%	1781	AB126653.1
Cystobasidium terricola strain CGMCC2.3824 18S small subunit ribosomal RNA gene, partial sequence; internal ...	Cystobasidium te...	1915	1915	95%	0.0	98.23%	2967	MK050391.1
Cystobasidium terricola strain CGMCC2.3823 18S small subunit ribosomal RNA gene, partial sequence; internal ...	Cystobasidium te...	1915	1915	95%	0.0	98.23%	2968	MK050390.1
Cystobasidium raffinophilum strain CGMCC2.3822 18S small subunit ribosomal RNA gene, partial sequence; int...	Cystobasidium r...	1915	1915	95%	0.0	98.23%	2967	MK050389.1
Cystobasidium sp. culture CPCC<CHN>:300514 small subunit ribosomal RNA gene, partial sequence	Cystobasidium sp.	1915	1915	95%	0.0	98.23%	1763	ON007439.1
Cystobasidium sp. culture CPCC<CHN>:300343 small subunit ribosomal RNA gene, partial sequence	Cystobasidium sp.	1915	1915	95%	0.0	98.23%	1752	ON007438.1
Cystobasidium sp. strain NYNU 21312 small subunit ribosomal RNA gene, partial sequence	Cystobasidium sp.	1915	1915	95%	0.0	98.23%	1737	OP218270.1
Cystobasidium minutum strain DBSC-5 18S ribosomal RNA gene, partial sequence	Cystobasidium m...	1912	1912	95%	0.0	98.31%	1698	FJ196163.1
Cystobasidium slooffiae strain DBSC-9 18S ribosomal RNA gene, partial sequence	Cystobasidium sl...	1912	1912	95%	0.0	98.31%	1702	EU978452.1
Cystobasidium slooffiae strain SA515 small subunit ribosomal RNA gene, partial sequence	Cystobasidium sl...	1911	1911	94%	0.0	98.56%	1660	MK990645.1
Cystobasidium minutum isolate T 18S ribosomal RNA gene, partial sequence	Cystobasidium m...	1911	1911	95%	0.0	98.14%	1236	AY150055.1
Cystobasidium minutum strain MCA4210 small subunit ribosomal RNA gene, partial sequence	Cystobasidium m...	1909	1909	93%	0.0	98.82%	1653	MK990643.1
Cystobasidium sp. 'nymphaeae' gene for 18S rRNA, partial sequence, strain:18-2A(IAM14903)	Cystobasidium s...	1907	1907	95%	0.0	98.14%	1736	AB055189.1
Cystobasidium oligophagum 18S rRNA gene, partial sequence; from TYPE material	Cystobasidium ol...	1906	1906	95%	0.0	98.05%	1750	NG_063083.1
Cystobasidium sp. 'cassiicola' gene for SSU rRNA, partial sequence, strain:36-2A(IAM14905)	Cystobasidium s...	1906	1906	95%	0.0	98.05%	1742	AB055191.1
Uncultured Basidiomycota clone North_Pole_SI120_110 18S ribosomal RNA gene, partial sequence	uncultured Basidi...	1903	1903	94%	0.0	98.47%	1607	JF826325.1
Cystobasidium calyptogenae strain SA628 small subunit ribosomal RNA gene, partial sequence	Cystobasidium c...	1902	1902	94%	0.0	98.38%	1665	MK990646.1
Cystobasidium calyptogenae gene for 18S rRNA, partial sequence	Cystobasidium c...	1902	1902	95%	0.0	98.05%	1782	AB126648.1

Figure 13: *Overview of BLAST hits of the 18S sequence obtained showing similarity to Cystobasidium.*

In order to achieve a complete genome sequence and identify the species with greater accuracy, Nanopore MinION sequencing was utilized. Despite efforts, the sequencing run did not produce the expected results, as shown in **Figure 14**.

Figure 14: *Run report from the Nanopore MinION run.*

For the biological characterization of the unicellular host an adapted chemotaxis assay based on capillarity was developed to confirm the movement of the cells in the culture. The outcomes for the this are shown in **Table 11** . After exposing the cells to different conditions, the content of each pipet tip was carefully dispensed in the microwell plates and diluted. For the next days the presence/absence of cells were verified by microscopy. Any well with cells was considered positive. Note that cells were detected in much higher dilutions of the 50% PYG condition, indicating that more cells entered the pipet tip containing this media than in the others, a clear indication of positive chemotaxis. A graphical representation of inverse of last dilution with respect to cells is given in **Figure 15**. The bar chart indicates highest growth at 50% PYG media.

Table 11: *Chemotaxis assay results. Wells with cell growth detected are color coded in green.*

	1	2	3	4	5	6	7	8	9	10	11	12
A	1% mucin											
B												
C												
D	MY media											
E												
F	50% PYG											
G												
H												
Dilution	**none**	**1/2**	**1/6**	**1/18**	**1/54**	**1/162**	**1/486**	**1/1458**	**1/4374**	**1/13122**	**1/30366**	**1/188098**

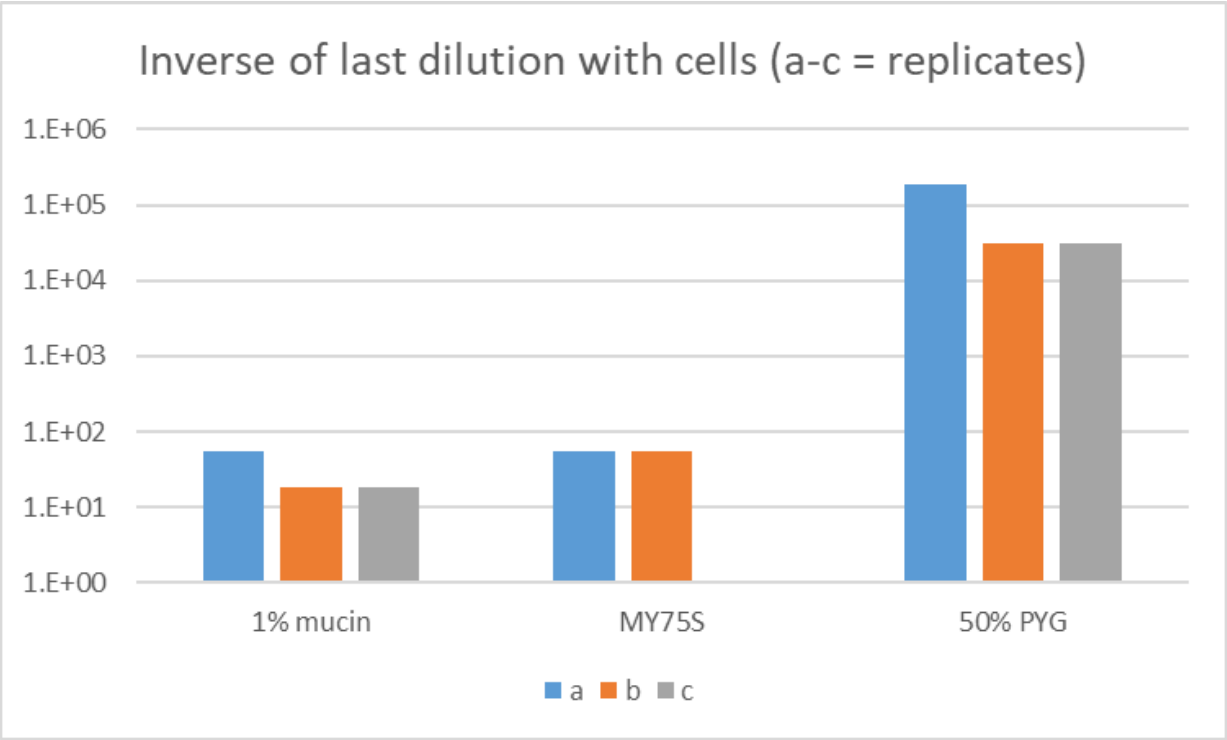


Figure 15: *Graphical representation of the chemotaxis assay results. For each replicate we plotted the inverse of the last dilution in which cells were detected.*

4.3 Virus isolation from arctic samples

In order to isolate viruses, the screening of the samples was carried out in two phases. Firstly, the samples were tested on *Acanthamoeba*, which served as a model host. Subsequently, the samples were tested on the arctic unicellular eukaryote, *Cystobasidium*. In principle we expected to find conventional giant viruses using *Acanthamoeba* and novel mycoviruses using the yeast.

4.3.1 Virus isolation in Acanthamoeba

We conducted experiments utilizing two species of *Acanthamoeba*, which are commonly used as model organisms. The distribution of the 96 well plate is illustrated in **Table 9**, and the outcome from the first passage is depicted in **Figure 16**. However, we encountered challenges related to contamination that persisted from the 1st passage until the 3rd passage, as can be observed in **Figure 17**.

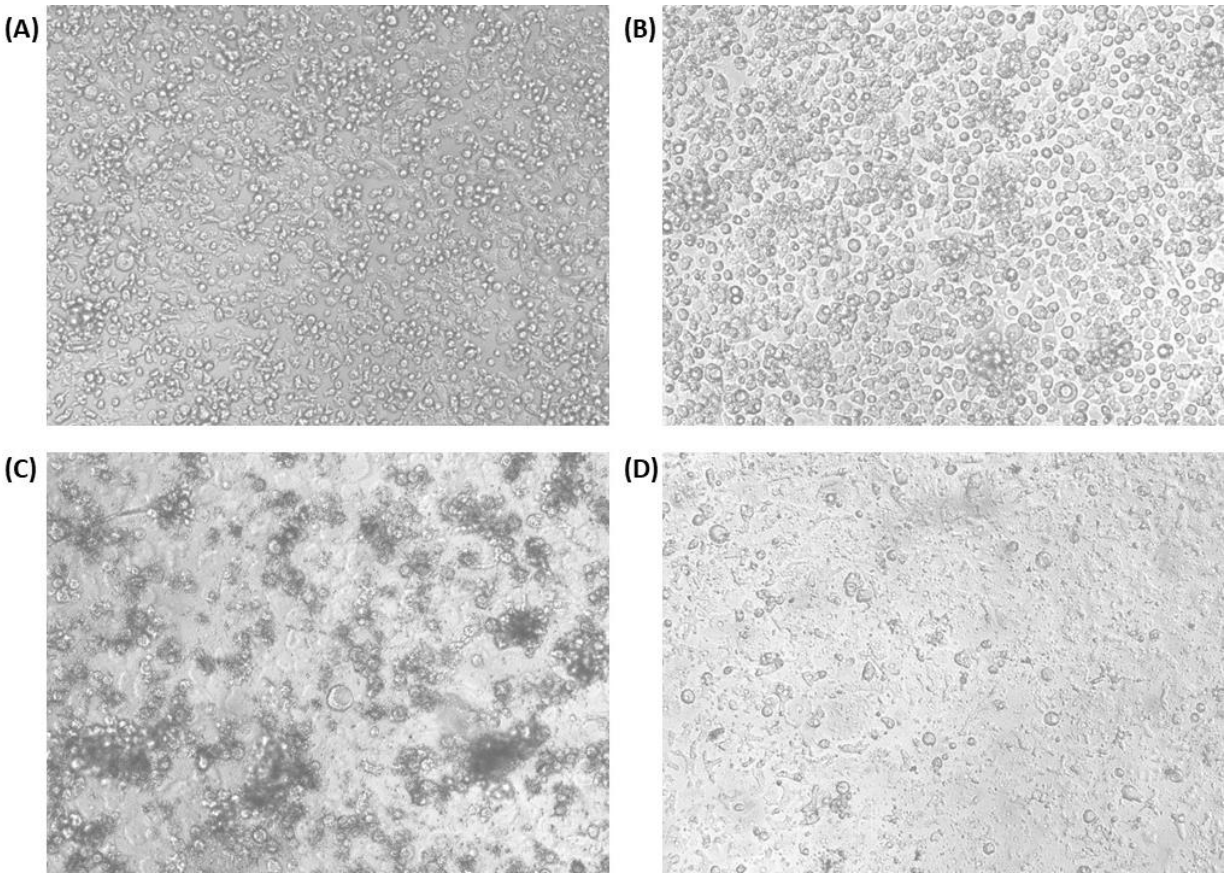


Figure 16: *Passage 1 of Acanthamoeba (A) A. castellani control and (B) A. polyphaga control (C) bacterial contamination in sample 58 in A. polyphaga, (D) A.polyphaga exposed to sample 39. Pictures taken with a 200x magnification.*

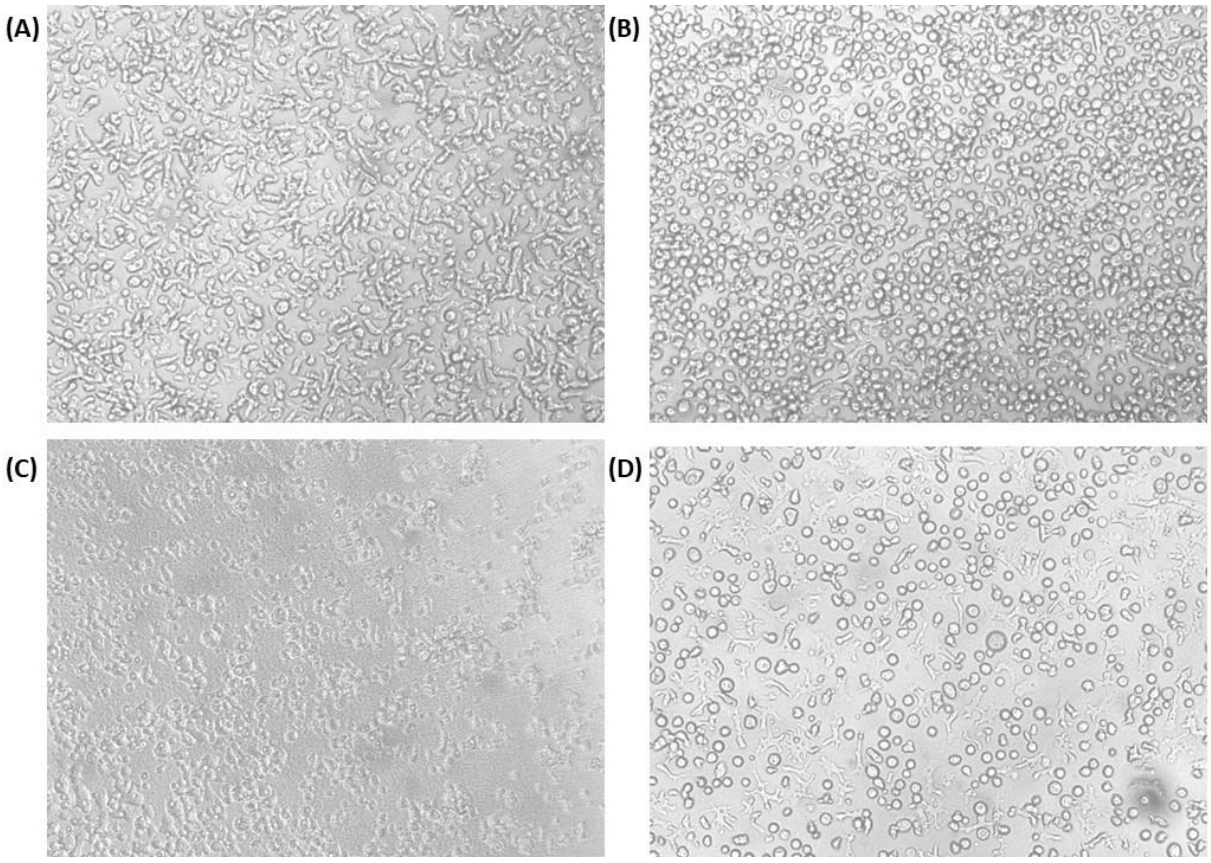


Figure 17: *Passage 3 of Acanthamoeba (A) A. castellani control and (B) A. polyphaga control (C) bacterial contamination in sample 28 (D) sample 80 led to some cell death. Pictures taken with a 200x magnification.*

4.3.2 Virus isolation in Cystobasidium sample testing

Following the isolation and characterization of the *Cystobasidium*, we progressed with the screening process for viruses in a 96 well plate. The distribution of samples across the plate is illustrated in **Table 10**. To confirm the cytopathic effects, we repeated the process for five passages. In the first passage **Figure 18**, control cells are shown in (A), while other pictures (B), (C), and (D) showed different growth patterns compared to the control. In passage 2, as demonstrated in **Figure 19**, we observed some clumping in (B), which was a different behavior from control (A); (C) and (D) showed some contamination. The subsequent passage, as depicted in **Figure 20**, revealed clear clumping signs, potentially caused by the potential virus. We proceeded with the most promising samples, namely, 33, 54, and 75, to passage 5 (**Figure 21)**, where they continued to exhibit the clumping phenomenon. In contrast, the control group showed no signs of clumping. Therefore, we prepared these samples for Transmission Electron Microscopy (TEM) analysis.

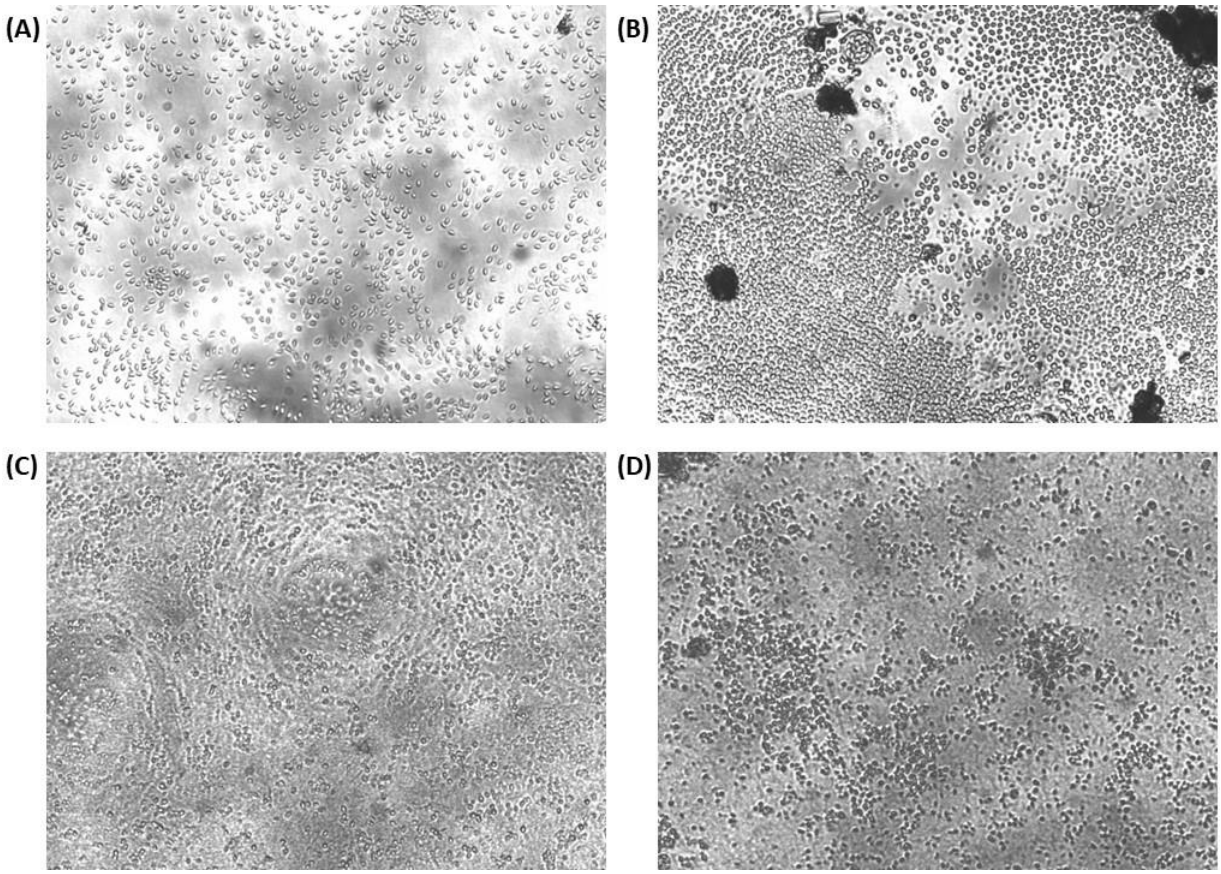


Figure 18: *Passage 1 of virus isolation using Cystobasidium as host. (A) control cells, (B) cells exposed to sample 60, (C) cells exposed to sample 16, and (D) cells exposed to sample 25. Pictures taken at 200x magnification.*

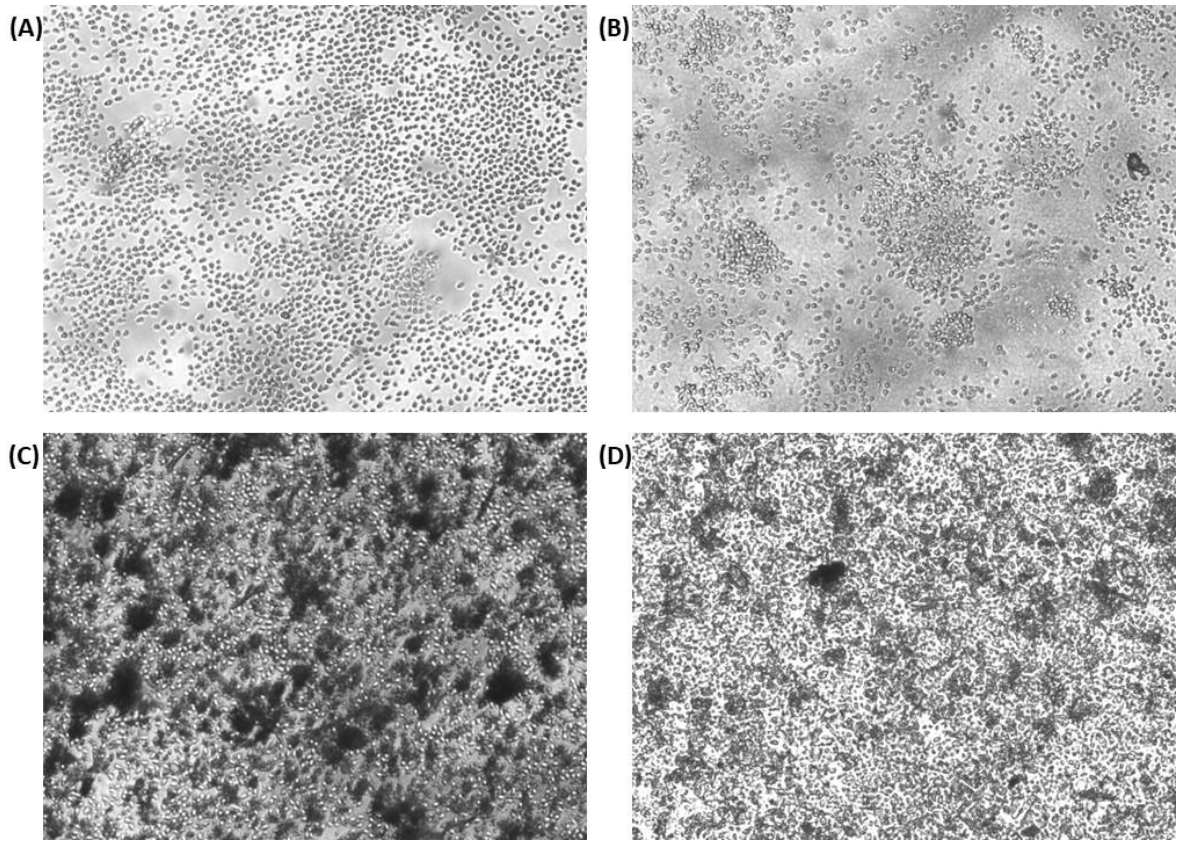


Figure 19: *Passage 2 of virus isolation using Cystobasidium as host. (A) control cells, (B) cells exposed to sample 35 - CPE, (C) cells exposed to sample 16 - contamination, and (D) cells exposed to sample 25 - contamination. Pictures taken at 200x magnification.*

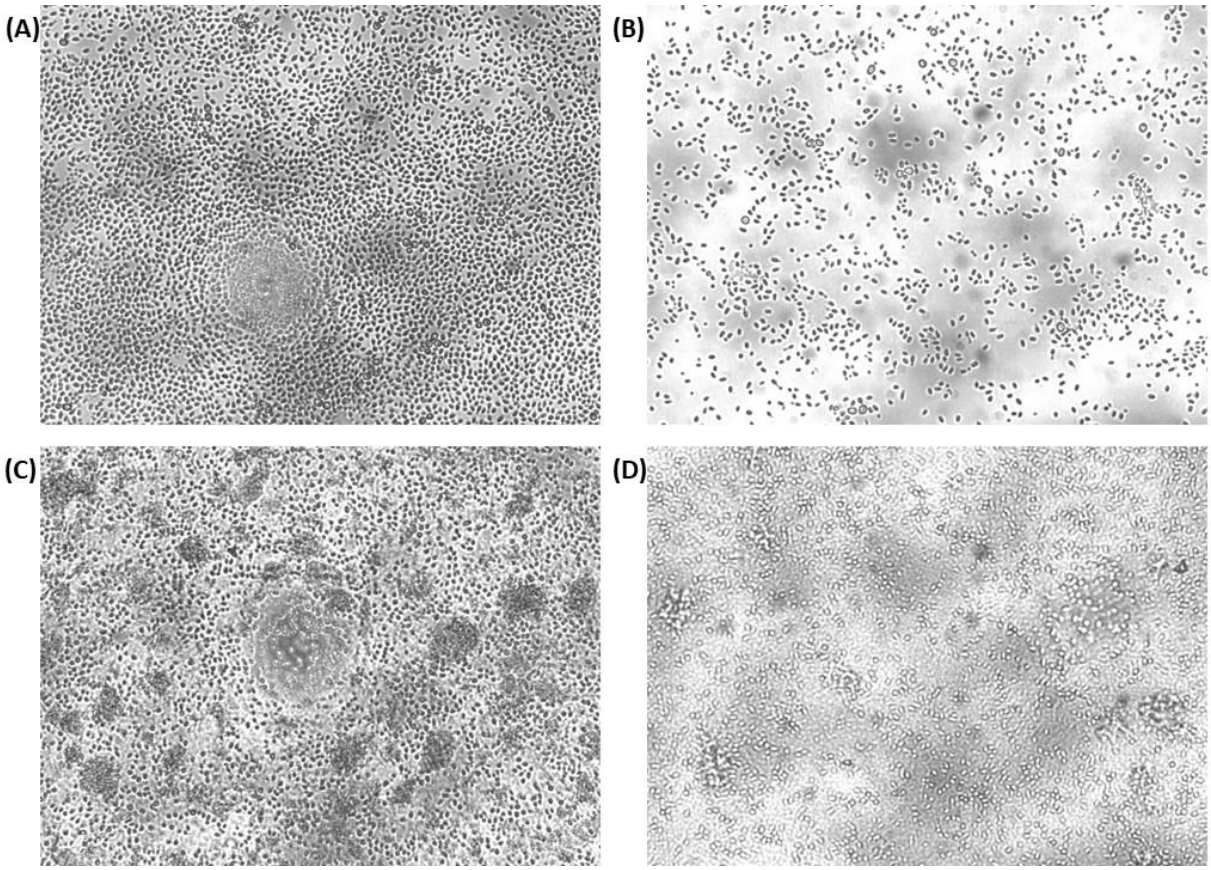


Figure 20: *Passage 3 of virus isolation using Cystobasidium as host. (A) control cells, (B) cells exposed to sample 33 - CPE, (C) cells exposed to sample 54 - CPE, and (D) cells exposed to sample 75 - CPE. Pictures taken at 200x magnification.*

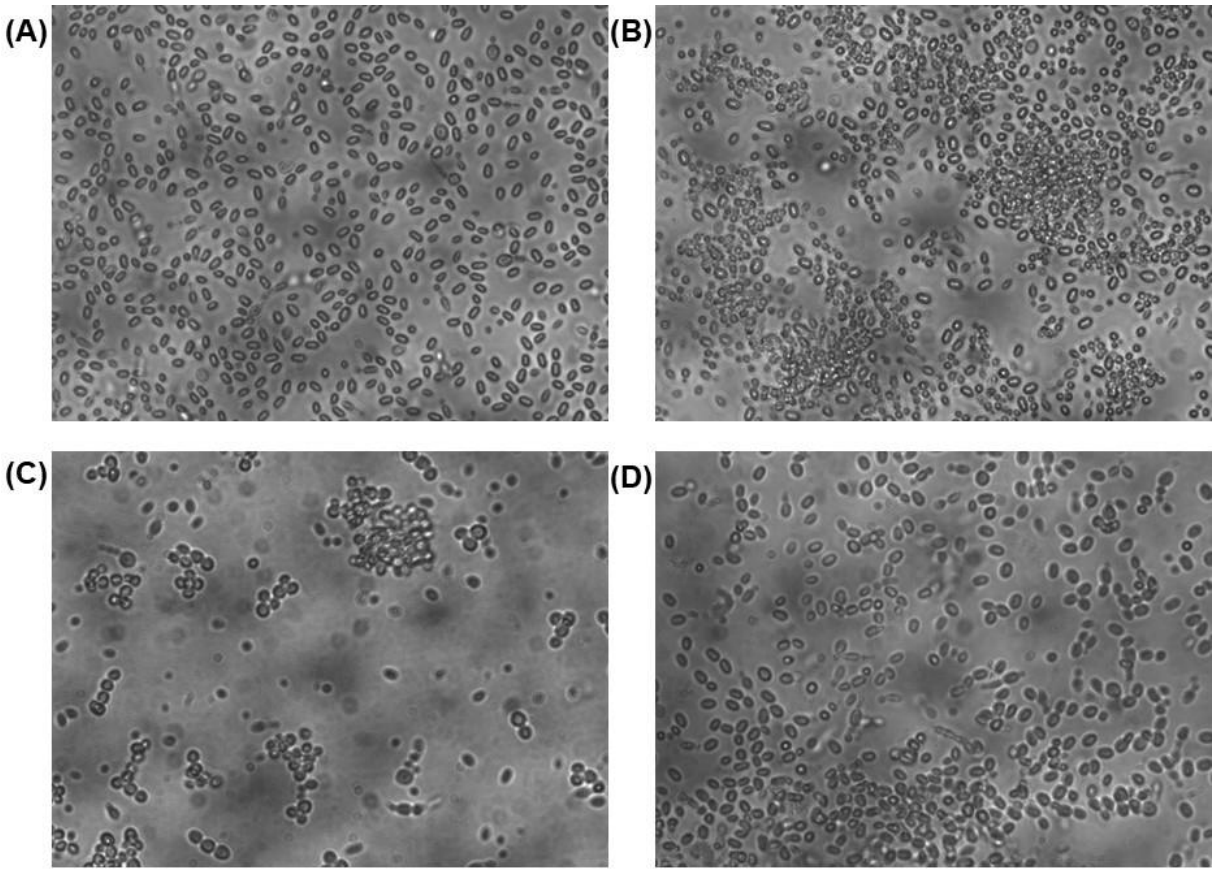


Figure 21: *Passage 5 of virus isolation using Cystobasidium as host. (A) control cells, (B) cells exposed to sample 33 - CPE, (C) cells exposed to sample 54 - CPE, and (D) cells exposed to sample 75 - CPE. Pictures taken at 400x magnification.*

4.3.3 Virus isolation and outcomes

A compilation of all the virus isolation attempts is shown in **Table 12**. Note that many samples were considered positive in the *Cystobasidium* host. We decided to follow up with sample 75, since it is from an interesting origin (deep sea) and presented clear CPE.

Table 12: *List of samples tested on different hosts and their outcomes.*

Sample#	**Results in *Acanthamoeba castellanii***	**Results in Acanthamoeba polyphaga**	**Results in *Cystobasidium***
1	Not tested	Not tested	Negative
2	Not tested	Not tested	Negative
3	Not tested	Not tested	Negative
4	Not tested	Not tested	Negative
5	Not tested	Not tested	Negative
6	Not tested	Not tested	Negative
7	Not tested	Not tested	Negative
8	Not tested	Not tested	Negative
9	Not tested	Not tested	Negative
10	Not tested	Not tested	Negative
11	Not tested	Not tested	Negative
12	Not tested	Not tested	Negative
13	Not tested	Not tested	Negative
14	Not tested	Not tested	Negative
15	Not tested	Not tested	Negative
16	Not tested	Not tested	Contamination
17	Not tested	Not tested	Negative
18	Not tested	Not tested	Negative
19	Not tested	Not tested	Negative
20	Not tested	Not tested	Negative
21	Not tested	Not tested	Negative
22	Not tested	Not tested	Negative
23	Not tested	Not tested	Negative
24	Not tested	Not tested	Negative
25	Not tested	Not tested	Contamination
26	Not tested	Not tested	Negative
27	Not tested	Not tested	Negative
28	Contamination	Contamination	Negative
29	Negative	Negative	Negative
30	Negative	Negative	Negative
31	Negative	Negative	Negative
32	Negative	Negative	Negative
33	Negative	Negative	Positive
34	Contamination	Contamination	Negative
35	Contamination	Contamination	Negative
36	Negative	Negative	Negative
37	Negative	Negative	Negative
38	Negative	Negative	Negative
39	Contamination	Contamination	Negative
40	Negative	Negative	Negative
47	Negative	Negative	Negative
48	Contamination	Contamination	Negative
49	Negative	Negative	Negative
50	Negative	Negative	Negative
51	Contamination	Contamination	Negative
52	Contamination	Contamination	Negative
53	Negative	Negative	Negative
54	Negative	Negative	Positive
55	Contamination	Contamination	Negative
56	Contamination	Contamination	Negative

57	Negative	Negative	Negative
58	Negative	Negative	Negative
59	Not tested	Not tested	Negative
60	Not tested	Not tested	Negative
61	Not tested	Not tested	Negative
62	Not tested	Not tested	Negative
63	Not tested	Not tested	Negative
64	Not tested	Not tested	Negative
65	Not tested	Not tested	Negative
66	Not tested	Not tested	Negative
67	Not tested	Not tested	Negative
68	Not tested	Not tested	Negative
69	Not tested	Not tested	Negative
70	Not tested	Not tested	Negative
71	Not tested	Not tested	Negative
72	Not tested	Not tested	Negative
73	Not tested	Not tested	Negative
74	Not tested	Not tested	Negative
75	Not tested	Not tested	Positive
76	Not tested	Not tested	Negative
77	Not tested	Not tested	Negative
78	Not tested	Not tested	Negative
79	Not tested	Not tested	Negative
80	Contamination	Contamination	Negative
81	Contamination	Contamination	Negative
82	Negative	Negative	Negative
83	Negative	Negative	Negative
84	Contamination	Contamination	Negative
85	Contamination	Contamination	Negative

4.3.4 TEM visualization of infected cells

Electron microscopy is a powerful tool that allows us to visualize the structure of various microorganisms. Among these, *Cystobasidium* stands out due to its remarkably thick cell wall. In **Figure 22**, we observe the control (A) and (B) images, which demonstrate the thick cell walls of *Cystobasidium* cells. On the other hand, images (C) and (D) display the sample 75, where unidentified extracellular particles are visible, warranting further investigation and analysis. Sadly, the thick cell walls prevented proper fixation of the intracellular environment, making it hard to say whether viral-like particles were inside the cells. The structures seen in the extracellular environment were diverse and some had virus-like morphology. Although bacterial contamination cannot be ruled out, if these are bacterial cells then they are small and resistant to the antibiotics used. No extracellular structures at all were seen on the control cells.

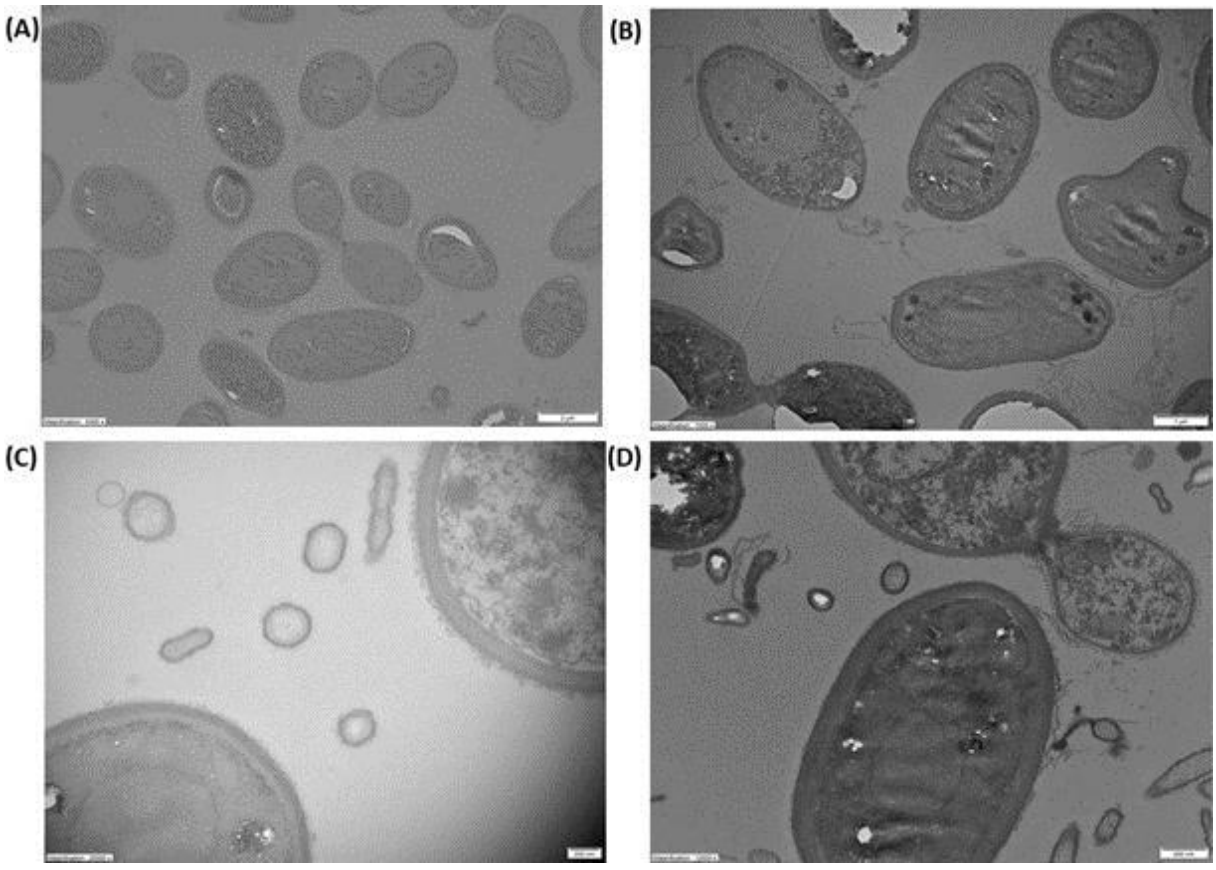


Figure 22: *TEM of virus isolation using* Cystobasidium *as host. (A)(B) control cells. (C)(D) cells exposed to sample 75 (fifth passage). Magnification is indicated on the figures by a size bar.*

5 Discussion

The discovery of a giant virus in 2003 was a pivotal event in the field of virology, as it challenged the existing paradigm of viruses as small, filter-passing particles. The first giant virus of amoeba, *Acanthamoeba polyphaga mimivirus* (APMV), also known as *mimivirus*, not only expanded our understanding of viruses, but also raised questions about the diversity of giant viruses waiting to be discovered. Despite the ubiquity of viruses in nature, little is known about the real diversity of giant viruses. Although many have been discovered in different parts of the world, there is a research gap to be filled when considering extreme environments and hosts outside the conventional domain. The present study aims to discover an arctic virus using an arctic host, with the understanding that arctic viruses likely have a host in the same region. Eventually, current thesis serves two purposes, the discovery of new cellular life in the arctic, and its subsequent use as a host to find arctic viruses. The findings from the current project will contributes to a better understanding of the arctic microbiome and its potential applications.

At the outset of the experiment, samples were obtained from the aquaculture and environmental group, UiT. One of the samples consisted of fish scales on which some amoeba growth was observed. Isolation efforts were divided into two methods. First isolation attempt utilized NNA plate and dead *E. coli* as a food source. Results showed bacterial contamination which exhibited degradation of agar. It is unlike that the contamination on the sample came from mishandling or human error of the experiment, since aseptic measures have been taken carefully following the guidelines (Bykowski & Stevenson, 2008). Some of the reasons that might have triggered contamination could be *E. coli* that was not fully dead during the experiment, or it could be some bacteria presented in the sample that was resistant to the prior antibiotic treatment. The second attempt to isolate the amoeba utilized media to grow the sample in a cell culture plate, it failed to grow under the lab conditions. It was observed as soon as the fish cells disappeared the growth of amoeba stopped. These results make sense, since the microorganism we were trying to isolate might have been dependent on its food source, which were fish cells. There has been previous evidence that *Acanthamoeba* growth may be dependent on fish cells (Lee et al., 2006). This is possible that microbe was too dependent on the fish cells, and thus we were not able to follow with it.

One sample out of many grew successfully under the lab conditions, and after experimenting with different growth media concentrations, it was observed that MY75S media supplemented with 10% PYG media at room temperature showed the optimal growth levels. The organism

was identified as a yeast related to *Cystobasidium* genus of yeasts, which was confirmed through 18S gene sequencing via Sanger sequencing. To best of our knowledge this was a new finding as *Cystobasidium* was previously discovered specifically as a part of gut microbiota of fish but not on the scales of the fish (Valderrama et al., 2021). There have been recent findings in the arctic region which isolated and characterized novel species of *Cystobasidium* (Turchetti et al., 2018). This discovery proposes that *Cystobasidium* genus still needs exploration and the *Cystobasidium* specie we have isolated might be a new addition to the genus.

Furthermore, to identify the cells to its species level we performed ITS PCR but there were no positive results. Primer selection might be a reason for the failure of PCR amplification. The primers used for this project were ITS4r and ITS5r. ITS Primer mismatch is a common problem in the field of mycology and there is an ongoing debate about what ITS region should be selected (Tedersoo & Lindahl, 2016). Another technique which was implemented to know the organism at species level was Nanopore MinION sequences. It can provide the whole genome sequence of the sample. We were not successful to get data out of the nanopore sequencing machine. At first, we thought about optimizing the DNA extraction and sequencing procedure. However, experience from other users in the research group revealed that the same problem was also happening with other people and other sample types when the same chemistry reagents and flowcells were used. This means that the problem is larger than expected and will need other solutions outside this thesis scope. This led us to spend more time and resources in the characterization of the newly identified mycological microorganism.

One surprising finding was that *Cystobasidium* cells exhibited a movement behavior, this phenotype in yeasts has not been explored yet. So far, there has been evidence reporting chemotaxis movement phenomenon in a yeast *Saccharomyces cerevisiae*, but chemotaxis was not measured (Ghose et al., 2021). Given only the preliminary capillarity chemotaxis assay done here it is still too bold to strongly state that we have found a yeast species capable of actively move towards nutrients. The characterization of the new specie is currently out of the scope of this thesis which will be further investigated in a future research project. As we have obtained a potential host to cultivate viruses, the next objective of the thesis project was to explore its potential as virus host.

For virus isolation 86 samples were collected from different locations in and around Tromsø were used to analyze. From these samples, 31 of 86 were initially tested on *Acanthamoeba polyphaga* and *Acanthamoeba castellanii*. However, we faced challenges with controlling the

contamination in these samples. The contamination might be due to the fact that raw samples were used that might had microbes which were resistant to the antibiotics present in the media. It was unlikely that the contamination on the sample came from mishandling of experiment or human error, since aseptic measures had been taken carefully following similar guidelines as Bykowski et al. 2008. As we have discovered a potential new host for giant virus cultivation, the first objective of the current thesis was accomplished. Then, the investigation followed with exploration of giant virus infection in *Cystobasidium*.

Cystobasidium was then tested with all the samples we collected through the course of this project. After five passages of *Cystobasidium* with 86 samples, only three samples were found to have potential infectious agents when tested. Then, our goal was to visualize the infectious agent with electron microscopy. Sample 75 was chosen as it had the most prominent effect on the *Cystobasidium* cells. Continuous evidence has suggested that deep sea vent is a suitable niche for a diverse variety of viruses (Cheng et al., 2022). Out of 3 samples that showed positive results, sample 75 was originated from deep sea vent. As well as it showed the most significant difference in the control and sample cells making it the best sample to move with further investigation. The thick cell walls made it impossible to observe the presence or absence of viral like particles in the cell cytoplasm. As well as different morphologies were seen on the extracellular environment of the cells exposed to sample 75 but not on the controls, indicating that we indeed have isolated another small biological entity. Its viral nature will be investigated as a follow-up to this project. Considering the sizes and the host, it can possibly be a mycovirus, but only after sequencing we will be sure of it. Sample 75 came from a deep-sea vent, an indication that this finding is likely unique. The current thesis provides with new insights into the realm of giant viruses. Giant viruses were traditionally thought to be only culturable in *Acanthameoba* but current study opens a new possibility of giant viruses having a diverse host range and moving towards a more complex host.

The future prospects of this project include characterization of the isolated unicellular eukaryote i.e., *Cystobasidium*, identification at the specie level and then focus on the chemotaxis behavior of the cells. Additionally, efforts to characterize sample 75 with more microscopic and genomic analysis.

6 Conclusion

The research outcomes of this thesis have shed light on a new yeast species that has been isolated from marine samples. This yeast species has demonstrated remarkable movement and chemotaxis abilities which was a novelty of this thesis project. Additionally, the microorganism yielded positive results when used as a host for virus isolation. The discovery of this potentially novel yeast specie has opened up new avenues for exploration that require further investigation. The next crucial step in this research is to conduct genomic sequencing to determine whether this newly discovered yeast species aligns with any previously undocumented *Cystobasidium* species. Furthermore, it is imperative to investigate whether a mycovirus has been isolated from the deep sea using sequencing techniques. Microscopy will also play a vital role in unraveling the structural intricacies of this newly discovered yeast and verifying its positive chemotaxis towards nutrients. Traditionally, acanthamoeba has been identified as the main host for the giant viruses. In the study focused on *Acanthamoeba*, the absence of positive hits for giant viruses does not necessarily mean that this line of inquiry is concluded. Another master thesis within our research group has yielded a discovery of a giant virus in a different sample subset, which is currently being pursued with active and ongoing efforts to delve deeper into this revelation. The collaborative nature of our investigations allows for a comprehensive understanding of the complex microbial interactions within marine environments.

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