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Screening of Arctic virome - A pilot study to delineate the viral diversity

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Original Article

Abstract

Viruses are the most abundant and genetically diverse acellular biological entities in the oceans. Extreme environments like Polar Regions represent an important source of both DNA and RNA viruses. This study is focused on the diversity of viruses in water samples collected from stations 4.1 and 5 in Kongsfjorden, Arctic. DNA viruses inhabiting both stations from Kongsfjorden were represented mainly by bacteria infecting Cyanophage and Roseophage, insect infecting Iridovirus, fish infecting Lymphocystis virus and human infecting Adenovirus. *Emiliania huxleyi* virus (EhV) infecting algae and the Torque virus infecting humans were detected from station 4.1 only, whereas ssDNA virus and Torno virus with genome size ranging from about 200 to 600 bp were detected only from station 5. The study exhibited limited RNA viral communities from both stations among which 3 different RNA viruses like Aichivirus, Vesivirus and Macronovirus were detected from station 5 of Kongsfjorden.

***Keywords**: Arctic virus, metagenomics, viral flocculation, Emiliania huxleyi virus, roseophage, cyanophage, Kongsfjorden*

Introduction

Kongsfjorden, a glacial fjord located on the west coast of Svalbard Archipelago is influenced by both Atlantic and Arctic water masses and thereby stands as a distinct ecosystem in the Arctic region (Svendsen *et al.,* 2002; Cottier *et al.,* 2005). Temperature in the Arctic Circle rises quickly, about three times faster than the rest of the world (Nordli *et al.,* 2014). Global climate change enhances the glacial retreat, reducing the sea ice coverage and thereby causing increased melt water input into the Arctic Ocean (Ostby *et al.,* 2017; Walczowski *et al.*, 2017). These melt water input alter the hydrography and biogeochemistry of fjords (Kanna *et al.,* 2018; Cape *et al.,* 2019). Microbes dormant for a long time, especially ancient viruses hidden in ice for centuries, leach into the oceanic waters due to the melting of ice. These viral particles become infectious just after their revival and can infect animals and humans through water bodies.

Besides their infectious mode on plants and animals, viruses infecting unicellular organisms play significant ecological roles in marine ecosystems (Middelboe and Brussaard, 2017). However, our knowledge regarding viral genomes is limited towards those isolated from a few culturable hosts and the viral genome diversity is still underrepresented in publically

available genome databases (Rohwer, 2003; Brum *et al*., 2015). Metagenomic studies are powerful to explore uncharacterized viral lineages from extreme environments like arctic habitats (De Corte *et al.,* 2011; Aguirre de Carcer *et al*., 2015). Genome level diversity of viruses have so far been explored from the metagenomes of double-stranded DNA (dsDNA) viruses (Emerson *et al*., 2012; Bellas *et al.,* 2015), RNA viruses (Culley *et al*., 2014) and single-stranded DNA viruses (Labonte and Suttle, 2013; Zawar-Reza *et al*., 2014).

Success of viral metagenomics, the study of uncultured viral nucleic acid sequences from diverse environmental samples, depends on several factors (Thurber *et al.,* 2009). Since the viral nucleic acid content is incredibly low, huge volumes of water samples were processed to attain adequate concentration of metagenomic DNA from various environments (Hata *et al*., 2015; Asami *et al*., 2016; Hjelmso *et al*., 2017). A variety of virus concentration methods were developed (Allander *et al*., 2011; Hall *et al.,* 2014; Solomon *et al.,* 2016). The performance and efficiency of various viral nucleic acid extraction kits have been assessed (Read, 2001; Burgener and Gilgen, 2003; Saeidi *et al.,* 2017; Zhang *et al*., 2018) and the efficacy of each protocol varies (Martinez *et al*., 2014).

Since there is no universal signature gene present in viruses, many different genes were assessed as potential group-specific signature genes (Rohwer and Edwards, 2002). Group specific marker genes developed for viral diversity analysis include those encoding structural proteins such as portal protein, major capsid protein, tail sheath protein, auxiliary metabolism genes such as *psbA*, *psbB* and *phoH* and several polymerase genes (Adriaenssens and Cowan, 2014). Diversity analysis based on these marker genes revealed a wide range of viruses including bacteriophages, cyanophages, nucleocytoplasmic large DNA viruses and also virophages infecting hosts like Proteobacteria, Cyanobacteria, Actinobacteria, Firmicutes, eukaryotic algae, amoebae and viruses (Adriaenssens and Cowan, 2014; Singh and Antony, 2015). With the help of group specific marker genes, current study explains a metagenomic scrutiny of viral communities prevailing in two stations (station 4.1 and 5) of Kongsfjorden, Arctic. This study validated the occurrence of cold active phages along with a group of infectious viruses in Kongsfjorden waters.

Material and methods

Sample collection and pre-processing of water sample

Surface sea water samples (20 litres) were collected from station 4.1 (79°01′49.7″N, 11°47′59.8″E) and station 5 (78°57′59.8″N, 11°45′48.9″E) of Kongsfjorden during Arctic Expedition 2015-16 (NCPOR) (Fig. 1). 20 litres of seawater has been pre-filtered through a series of filter membranes with various pore sizes (11 μm, 1.22 μm, 0.45 μm & 0.22 μm) to remove coarse debris and bacterial fraction using a peristaltic pump and the resulting filtrate was collected and subjected to flocculation.

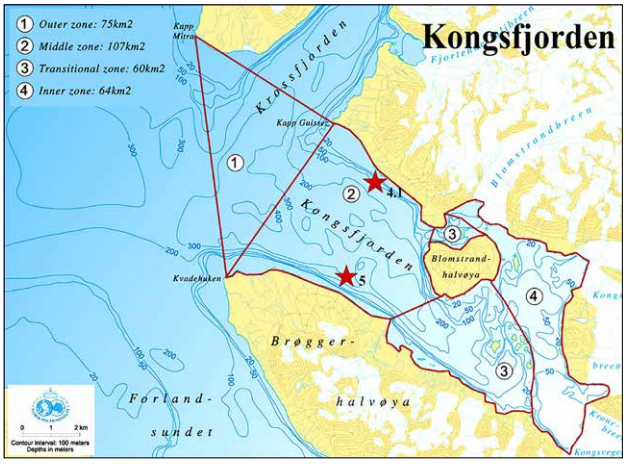

Fig.1. Map showing sampling sites (station no. 4.1 and station no. 5) from Kongsfjorden, Arctic (Source: Norwegian Polar Institute – www.npolar.no)

Flocculation and re-suspension of viral particles

$FeCl_3$-based virus flocculation was performed for the separation of virus particles from the filtrate (John *et al.*, 2011). In brief, 2 ml Fe solution (10g/L $FeCl_3$ stock solution) was added to the filtered water (20 litres), mixed thoroughly and incubated for a minimum of 1 hour at room temperature (28 ± 2 °C). After that, filtrate with virus flocculate was passed through 1.22 μm filter membrane (GE Healthcare life sciences, UK) and the filter paper with residue was kept in 50 ml centrifuge tubes and stored at 4 °C for further analyses. 1.5 ml of freshly prepared 0.2M Ascorbate-0.1M EDTA-Mg re-suspension buffer (pH: 6-7) was added into each tube and kept at 4 °C overnight in a shaker. After complete elution of precipitate from filter membranes, resuspension buffer containing the virus fraction was treated for viral nucleic acid extraction.

Viral nucleic acid extraction and quantification

Viral nucleic acids were extracted via QIAmp MinElute Virus Spin Kit (QIAGEN, Delhi) according to manufacturer's instructions. Quantity of extracted viral DNA and RNA were estimated using Qubit® dsDNA HS (High Sensitivity) assay kit and Qubit® RNA HS assay kit in a Qubit® Fluorometer as per manufacturer's directions.

Detection of viruses by amplification of viral DNA/ RNA targets

Primers specifically designed for DNA and RNA viruses were employed for PCR amplification (Singh and Antony, 2015). Viral nucleic acid was subjected to reverse transcription for cDNA synthesis from RNA using random primers. 20μl reverse transcription reaction mix containing 0.5μl of RNase inhibitor, 4 μl of RNA primer sets, 2μl dNTPs (10 mM), 1.6μl of $MgCl_2$ (25 mM), 2μl reverse transcriptase buffer (2X), 0.5 μl reverse transcriptase and 9.4 μl template. The reaction mix (20μl) after denaturation at 65 ˚C for 5 minutes was incubated at 42 ˚C for 1 hour followed by 15 minutes inactivation at 85 ˚C. Group-specific signature gene primers (37 sets) specifically designed for various marine viruses *viz*., Adenovirus, Anellovirus, Circovirus, *Chrysochromulina ericina* virus, Cyanophage, *Emiliania huxleyi* dsDNA virus, Iridovirus dsDNA, Lymphocystis virus dsDNA, Phycodnavirus, Roseophage, ssDNA virus, Tornovirus and Torque virus were used for this study.

All PCR reactions were performed in a 25μl reaction volume containing 1x standard Taq buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3), 2.5 mM $MgCl_2$, 200 μM dNTPs, 0.4 μM each primer and 1U Taq DNA polymerase (New England Biolabs, USA) and 1μl genomic DNA / cDNA template using Veriti™ 96-well thermal cycler (Applied Biosystems, USA). PCR conditions are 94 ˚C for 2 minutes (1 cycle), 94 ˚C for 15 seconds, 58 ˚C for 30 seconds, 68 ˚C for 30 seconds (35 cycles) and 68 ˚C for 10 minutes (1 cycle). Similar annealing temperature was applied for all primer sets except primers of iridoviridae (65 ˚C), phycodnaviridae (55 ˚C) and adenoviridae (60 ˚C) families. PCR products were electrophoresed in 1% agarose gel prepared in TBE (40 mM Tris-HCl, 20 mM Boric acid, 1 mM EDTA, pH 8.0), comprising ethidium bromide (0.5μg/ml) for nucleic acid staining and visualization (Gel Documentation System, SYNGENE, UK).

Cloning of PCR products

Purified PCR products were ligated into pGEM-T Easy vector (Promega) and ligated products were transformed into competent *Escherichia coli* DH5α cells. Transformed bacteria were cultured on Luria Bertani (LB) agar plates supplemented with 100 μg/ml of ampicillin, 80μg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) and 100mM isopropyl thio-β-D-galactoside (IPTG) and incubated at 37 ˚C overnight. Recombinant clones were selected by blue-white screening, colony PCR was carried out and plasmid isolation was performed using conventional method (Birnboim and Doly, 1979). Recombinant clones were screened *via* plasmid PCR using vector-specific (T7F and SP6R) and gene specific primers. PCR products were analysed by 1% agarose gel electrophoresis and plasmid sequencing was done at Scigenom, Cochin, India.

Plasmid sequencing and sequence analysis

The sequence obtained was screened for vector contamination in VecScreen software. Blast analysis was done in ViroBLAST to find out highly similar sequences. Relevant sequences of viruses were also retrieved from GenBank and multi-aligned using ClustalW. Phylogenetic tree was constructed by Maximum-Likelihood (ML) method using MEGA-X software.

Results and discussion

A key challenge to researchers in environmental microbiology has been collection, extraction and detection of viruses from large volumes of water samples. Though ≤ 0.02 μm filter membranes are used for virus filtration, low filtration speed and speedy clogging of these filters made this approach only applicable to smaller volumes of water samples. Considering this crisis, a number of techniques were developed to concentrate viral particles from larger volumes of water samples which include adsorption-elution methods (Kamata and Suzuki, 2003), ultracentrifugation (Colombet *et al*., 2007), vortex flow filtration (VFF) (Paul *et al*., 1991) and tangential flow filtration (TFF) (Wommack *et al*., 2010). Still, they have their own limitations with respect to the recovery of viruses. Though studies have proven TFF as the most efficient approach for larger volumes of virus recovery (Wommack *et al*., 2010), it requires lengthy processing time and expensive equipment. Considering the limitations of these approaches, John *et al.* (2011) developed an efficient and less expensive Fe-mediated flocculation method for virus concentration ensuring more than 90% viral recovery from ocean waters. This method is developed by adapting coagulation and flocculation based wastewater treatment techniques which were also employed for the virus concentration of soil, marine waters, sewage and effluents (Chaudhuri and Engelbrecht, 1970; Berg, 1973; Bitton, 1975; Han *et al.,* 2002; Wang *et al.,* 2018). Like other studies (Bellas *et al*., 2015; Nishimura *et al.,* 2017), the present study also has proven that flocculation is effective in the revival of viral particles from glacial and fjord water samples.

Viral nucleic acids could be extracted using QIAamp MinElute Virus Spin Kit (QIAGEN). PCR amplification of viral nucleic acids could be obtained for 12 sets of primers and showed the presence of 7 groups of marine viruses such as Adenovirus, Cyanophage, *E. huxleyi* dsDNA virus, Iridovirus dsDNA, Lymphocystis virus dsDNA, Roseophage and Torque virus in surface waters of station no. 4.1 (Table 1). Adenovirus, Lymphocystis virus dsDNA, Roseophage, Tornovirus and Cyanophage with the predominance of Iridovirus and ssDNA viruses were identified from station 5 (Table 2). Within these reported groups, *Emiliania huxleyi* dsDNA virus and Torque virus stand unique for station 4.1 while

Table 1. List of DNA viruses detected from station 4.1 in Kongsfjorden, Arctic

Sl. No.	Virus	Primers	Annealing Temperature (°C)	Product Size (bp)
1	Adenovirus	ADENO- DSD- F ADENO- DSD- R	60	200
2	Cyanophage	CYANO- PD- S- F CYANO- PD- S- R	58	200
3		CYANO- POL- F CYANO- POL- R	58	75
4		CYANO- G20- F CYANO- G20- R1	52	75
5	*Emiliania huxleyi* dsDNA virus	EMILI- DSD- F EMILI- DSD- R	55	200
6	Iridovirus	IRIDO-DSD- CAP-S- F IRIDO-DSD-CAP-S- R	61	350
7		IRIDO- TNFR1- F IRIDO- TNFR1- R	65	250
8		IRIDO- TNFR2- F IRIDO- TNFR2–R	65	500
9	Lymphocystis virus dsDNA	LYMPHO 2- DSD- F LYMPHO 2- DSD- R	50	150
10	Roseophage	ROSEO- PD- F ROSEO- PD- R	61	550
11		ROSEO- CAP1- F ROSEO- CAP1- R	50	75
12	Torque virus	TORQUE-2-DSD- F TORQUE-2-DSD- R	58	75

Table 2. List of DNA viruses detected from station 5 in Kongsfjorden, Arctic

Sl No.	Virus	Primers	Annealing Temperature (°C)	Product Size (bp)
1	Adenovirus	ADENO2-DSD-F ADENO2-DSD-R	61	200
2	Cyanophage	CYANO-PD-L- F CYANO-PD-L- R	64	400
3	Iridovirus	IRIDO-DSD-CAP-S-F IRIDO-DSD-CAP-S-R	61	300
4		IRIDO-DSD-MCP-L-F IRIDO-DSD-MCP-L-R	64	200
5		IRIDO- TNFR1- F IRIDO- TNFR1- R	65	250
6		IRIDO- TNFR2- F IRIDO- TNFR2–R	65	500
7	Lymphocystis virus dsDNA	LYMPHO3-DSD- F LYMPHO3-DSD- R	58	200
8	Roseophage	ROSEO-CAP2- F ROSEO-CAP2- R	58	600
9	ssDNA virus	SEN-SSD-F SS-SSD-R	64	300
10		SS-SSD-F SS-SSD-R1	55	400
11		SS-SSD-F SS-SSD-R2	52	200
12	Torno virus	TORNO-SSD-F TORNO-SSD-R	61	200

Tornovirus and ssDNA viruses were unique for station 5. Three groups of RNA viruses were detected from station 5 and none from station 4.1 (Table 3). Amplicons of these viral DNA/RNA inserts in plasmids are shown in Fig. 2 and the phylogenetic trees in Fig. 3 and 4.

Table 3. List of RNA viruses detected from station 5 in Kongsfjorden, Arctic

Sl No	Virus Name	Primer Name	Annealing Temperature (°C)	Product Size (bp)
1	Aichivirus	AICHI-SSR-F AICHI-SSR-R	55	400
2	Vesivirus	VESSI-SSR-CAP-5.6F VESSI-SSR-CAP-5.6R	55	200
3		VESSI-SSR-CAP-2.5F VESSI-SSR-CAP-2.5R	55	200
4	Macronovirus	MR-0.5-4F MR-0.5-4R	55	200
5		MR-0.45-5F MR-0.45-5R	55	75

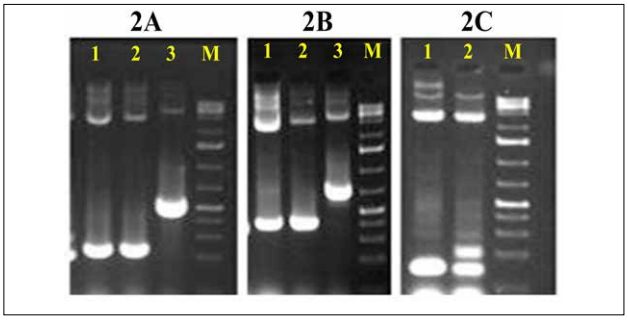

Fig. 2. Agarose gel of plasmid PCR cofirming the viral amplicon inserts. 2A & 2B: Gene specific & vector specific PCR gel image (Lane 1& 2: 250bp inserts of Adenovirus DNA, Lane 3: 550bp insert of Roseophage, Lane M: 1kb ladder; 2C: Plasmid PCR gel image (Lane 1 & 2: 150bp insert of Lymphocystis virus, Lane M: 1kb ladder).

From the ecological point of view, *Emiliania huxleyi* virus, Cyanophage and Roseophage stand distinctive within these marine virus groups. Molecular phylogeny inferred from the nucleotide sequences of signature genes established the occurrence of *E. huxleyi* virus (EhV) from station 4.1 (Fig. 3A). *E. huxleyi,* the photosynthetic unicellular eukaryote is the most dominant bloom-forming species of coccolithophores in the oceans and plays an important role in the cycling of nutrients and biogenic sulphur production in the form of dimethyl sulphide (Elderfield, 2002). *E. huxleyi* virus (EhV) is a lytic giant virus belonging to the genus coccolithovirus within the family phycodnaviridae which infects marine coccolithophorid *E. huxleyi.* The infection and subsequent lysis of *E. huxleyi* cells by EhV is the key mechanism causing the termination of algal blooms (Jacquet *et al.*, 2002; Lehahn *et al.,* 2014). The *E. huxleyi* cell death results in enhanced shedding of the virus into the seawater. In this regard, the largest sink of inorganic carbon on the earth is the outcome of a rapid crash of *E. huxleyi* blooms in the oceans during the EhV increase (Frada *et al.*, 2008). Studies have been already reported about the cloning and sequencing of amplified segments of the major capsid protein (MCP) gene from viruses that infect *E. huxleyi* (Schroeder *et al.*, 2002). EhV shows a wide distribution in subpolar waters and has a significant impact on the native population of the algae. The presence of EhV in Kongsfjorden water sample infer its enormous impact on biogeochemical cycles and host-virus dynamics in that environment.

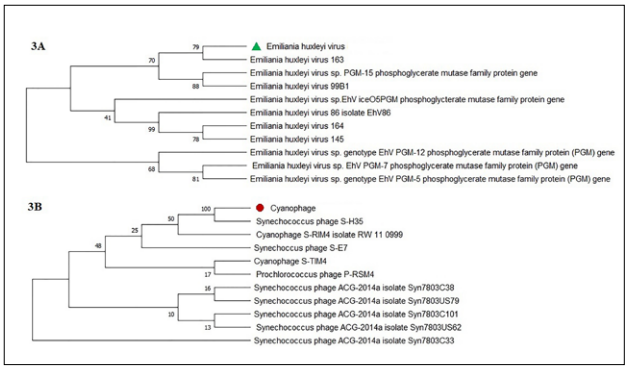

Fig. 3. Phylogenetic tree of *E. huxleyi* virus (3A) and Cyanophage (3B) detected from Kongsfjorden, Arctic. Phylogenetic trees were constructed using Maximum-Likelihood (ML) method using MEGA X.

Bacteriophages infecting Cyanobacteria (Cyanophages) and Roseobacter (Roseophages), which are abundant and play significant roles in modulating biogeochemical cycling, mediating genetic exchange and structuring ocean's microenvironment were also reported from both the stations of Kongsfjorden. Cyanobacteria are major primary producers in the aquatic environment and the abundance of Cyanophages can alter the metabolism and replication of their hosts, playing an important role in bloom dynamics and thereby regulating cyanobacterial community structure and photosynthesis (Xia *et al*., 2013; Roux *et al.,* 2016). Cyanophages were reported from all groups of Caudovirales like Myoviridae, Podoviridae and Siphoviridae (Ou *et al*., 2015a; Ou *et al*., 2015b). Structural genes like capsid protein gene (g20) and tail sheath protein (g91) from Myoviridae were used for PCR based detection of Cyanophages (Short and Suttle, 2005; Adriaenssens and Cowan, 2014). Since their occurrence has been detected from the Kongsfjorden waters, we can infer that Cyanobacteria occupy prime position in primary production in the area (Fig. 3B).

Roseobacter clade contributes a large proportion of the bacterioplankton population in the coastal and open ocean, and some Roseobacters are found to be associated with microalgae, particularly during phytoplankton blooms (Buchan *et al*., 2014). By affecting the fate of DMSP, Roseobacters can influence the global sulphur cycle (Howard *et al.,* 2008; Vila-Costa *et al*., 2014). Studies proved that, along with Roseobacter lineage, bacteriophages infecting Roseobacters (Roseophages) also have significant biochemical roles in the marine ecosystem. While considering the knowledge regarding the genome sequences of Roseobacters, more than 77 genomes of marine Roseobacter species are already available (Zhang *et al.,* 2016; Simon *et al*., 2017). On the other hand, due to the lack of signature genes, very limited number of Roseophages have been isolated and characterized from different environments, including cold active Antarctic lakes (Yau *et al.,* 2013; Zhan *et al*., 2015; Zhan *et al.,* 2016). Using a pair of group-specific primers based on the DNA polymerase gene, Roseophages were detected in the Kongsfjorden waters. The findings from this study will allow us to explore further the phylogenetic diversity of Roseophages thereby gaining new insights into phage diversity and phage -bacterial host relationships (Cai *et al*., 2019).

Iridoviruses, a group of nucleocytoplasmic DNA viruses, act as emerging infectious disease agents, thereby causing great economic losses in the aquaculture industry and significant threat to global biodiversity (Wang *et al*., 2014). Iridoviruses are capable of infecting cold-blooded vertebrates, invertebrates, crustaceans and molluscs (Chinchar *et al.,* 2011). Major capsid protein (MCP) gene is mainly used for the identification and classification of Iridoviruses (Williams, 1994). In this study, Iridoviruses were detected from both stations in Kongsfjorden waters. Lymphocystis virus, coming under the family Iridoviridae is an important fish pathogen which can infect marine and freshwater fishes worldwide (Xu *et al*., 2014). Molecular screening using the major capsid protein (MCP) and DNA polymerase based primers revealed the occurrence of lymphocystis virus from both stations (Fig.4A). A phylogenetic tree of lymphocystis virus was constructed by the Maximum-Likelihood (ML) method using Molecular Evolutionary Genetics Analysis (MEGA-X). Phylogenetic analyses of these viruses strongly supported their existence in Arctic habitats.

The frequent occurrence Adenoviruses and Torque viruses in many aquatic environments is caused by human activities like sewage leakage, vessel water discharge, agricultural runoff and urban runoff (Lee and Kim, 2002). Adenoviruses infect a wide range of species, including humans, other mammals, birds, reptiles, amphibians and fishes. PCR based techniques were employed to find out the presence of these enteric viruses in

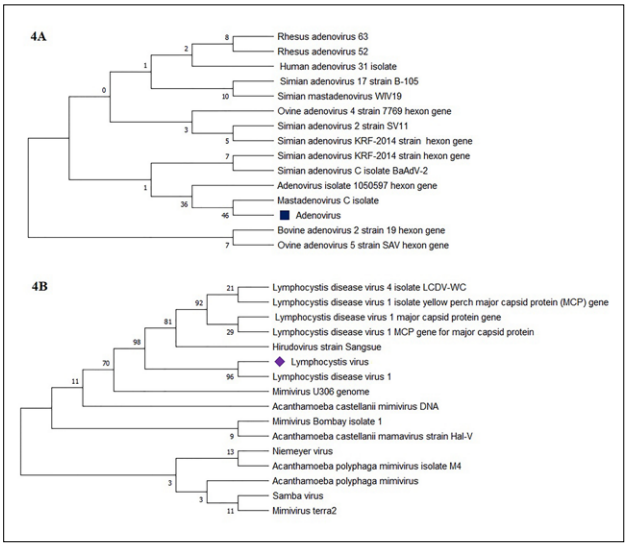

Fig. 4. Phylogenetic tree of Adenovirus (4A) and Lymphocystis virus (4B) detected from Kongsfjorden, Arctic. Phylogenetic trees were constructed using Maximum-Likelihood (ML) method using MEGA X.

marine environments (Muscillo *et al.,* 1994; Puig *et al.*, 1994). From Kongsfjorden waters, we have detected the presence of adenoviruses which may be due to the increased human activities in arctic habitats (Fig. 4B). Torque viruses, generally used as faecal contamination marker, were detected from station 4.1 and were frequently found in waters influenced by waste discharges more than in surface waters. Data limitations regarding torque viruses demonstrate that additional research is needed on this potential viral indicator (Charest *et al.,* 2015). Their presence in Kongsfjorden waters indicated the influence of increased human activities in arctic habitats.

Studies regarding the isolation and molecular analyses of marine viruses impart information about their possible infection on a diverse range of marine host species and contributions to the dynamics of marine ecosystems (Lang *et al.*, 2009). Another enteric virus, a member of the family Picornaviridae, Aichivirus (AiV) was detected in the present study. PCR based screening by targeting the RNA polymerase gene showed that AiV viruses are present in station 5 of Kongsfjorden indicating contamination in the water body. Presence of enteric viruses in arctic habitats indicated the release of human viruses into this environment. Vesiviruses in the family Caliciviridae, commonly known as sealion viruses, are known to infect a variety of marine species, including mammals, birds, fishes and invertebrates (Van Bressem *et al.*, 1999; Smith, 2000). Vesiviruses are infectious agents of migratory birds and mammals and are able to jump between marine and terrestrial hosts; there is a great opportunity of virus transmission to new locations (Lang *et al.*, 2009). Studies reported their infectious routes from whales, shellfishes and fishes to humans through marine systems (Smith, 2000; Martella *et al.,* 2015). Along with Aichivirus and Macronovirus (Family:Sarthroviridae), Vesiviruses also indicated their presence in Kongsfjorden waters. The findings of unique ssDNA viruses like Tornoviruses and RNA viruses from station 5 will contribute greatly to our understanding of the diversity of viruses inhabiting Arctic habitats.

Conclusion

PCR based detection of viruses using gene specific primers for DNA polymerase, major capsid proteins, hexon protein and tumor necrosis factor receptor were used in the present study to unravel the viral diversity in water samples collected from Kongsforden, Arctic. The study revealed that the viral communities in the Kongsfjorden waters comprised of Cyanophage, *E. huxleyi* dsDNA virus (EhV), Adenovirus, Iridovirus, Lymphocystis virus, Roseophage, Torno virus and Torque virus. Further studies on viral communities inhabiting Arctic environment with respect to host-virus interactions and their potential impact on global biogeochemical cycles would be highly promising.

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