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Soil Virus Ecology: Research Methods (Postprint)

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Date: 2017-04-11T00:00:00+00:00

Abstract

Viruses are the most abundant biological entities on Earth, with a single gram of soil potentially containing hundreds of millions of viruses. They not only influence the community composition of other microorganisms in soil and the biogeochemical cycling of soil elements, but also affect the speciation and evolution of soil microorganisms, and even impact plant, animal, and human health. Currently, our understanding of the types, abundance, distribution characteristics of soil viruses, and the ecological and environmental effects caused by their functions remains very limited. Based on an overview of research methods in viral ecology, this study conducts a comparative analysis of the basic procedures for soil virus extraction, purification, quantification, and molecular ecological methods, aiming to establish a rapid, simple, efficient, and stable methodology suitable for soil virus research. This methodology will be applied to investigate the diversity and distribution characteristics of soil viruses, explore the survival and transmission mechanisms of viruses in the environment, and provide support for the prevention, control, and utilization of soil viruses.

Full Text

Preamble

ACTA ECOLOGICA SINICA (ChinaXiv Partner Journal)
Vol. 37, No. 6, March 2017
DOI: 10.5846/stxb201511229292

Research Methods for Soil Viral Ecology

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Abstract

Viruses are the most abundant biological entities on Earth, with concentrations reaching up to 10 viral particles per gram of soil. They influence the composition of microbial communities, mediate soil biogeochemical cycles, regulate microbial evolution, and affect the health of plants, animals, and humans. However, knowledge remains limited regarding the abundance and distribution patterns of soil viruses and their ecological impacts. This study provides a comparative analysis of methods for soil viral research, including extraction, purification, quantification, and molecular ecology techniques. The development of reliable and highly efficient methods for evaluating soil viral particles is essential for investigating viral diversity and distribution, understanding viral propagation mechanisms in soils, and informing strategies for viral control and public health protection.

Keywords: soil virus; viral ecology; metaviromics; molecular biological methods

Introduction

Viruses are the most abundant biological entities on the planet and play crucial roles in soil nutrient biogeochemical cycling, microbial community composition, and genetic resource diversity. Viruses infect microorganisms such as bacteria and cyanobacteria, affecting their physiological metabolism and thereby influencing microbial community structure and diversity in ecosystems. Cell lysis caused by viral infection releases organic matter that can be absorbed by other microorganisms through transcription, affecting nutrient utilization and biogeochemical cycling of elements. The high heterogeneity of soil and its diverse biological environment provide rich and varied habitats for viruses, facilitating their long-term survival and reproduction.

Despite their important ecological functions, research on soil viruses has progressed slowly due to their small size, low genomic content, lack of universal genes, and short life cycles. Viral ecology research has evolved through several key stages: phenotypic analysis, genomic analysis, and metagenomic analysis. Scientists have used microscopy to study viruses and bacteria in extreme environments such as the Sahara and Namib deserts, revealing unique bacteriophage communities. More recent studies have combined phenotypic and molecular biological methods, such as transmission electron microscopy (TEM) and pulsed-field gel electrophoresis (PFGE), to investigate both isolated phages and viral community composition. Genome-level studies have revealed that some marine

phage genomes appear only at specific locations, while others are widely distributed. Research on vibriophages has shown that abundance and geographic distribution are not directly correlated but are instead driven by habitat type, including associations with sediments and invertebrates.

The advent and widespread application of sequencing technologies has overcome previous bottlenecks in environmental microbiology research. Viral genome sequencing began with ΦX174, a phage infecting *E. coli*. In 2002, Breitbart et al. first used shotgun sequencing to study marine viral metagenomes. In 2005, Edwards and Rohwer proposed the concept of viral metagenomics, and in 2014, Hurwitz et al. used comparative genomics and network analysis to model ecological drivers of marine viral communities. Bolduc et al. applied network analysis to reveal viral community composition and interactions in Yellowstone acidic hot springs. However, compared to seawater and hot spring samples, soil samples present greater challenges for virus extraction due to their high heterogeneity and complex composition.

This paper reviews advances in viral ecology research methods and establishes a rapid, simple, efficient, and stable methodology for soil viral ecology studies. The approach includes viral sample enrichment, fingerprint analysis, and metagenomic sequencing.

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1. Extraction of Soil Viruses

Virus adsorption to soil is closely related to virus type and environmental factors such as soil structure. The first step in soil virology research is selecting an appropriate and efficient extraction method to effectively recover viral particles adsorbed to soil surfaces. Common extraction solutions include 250 mmol/L glycine solution, 10 mmol/L glycine solution, potassium citrate solution, sodium pyrophosphate, and beef extract. Studies have compared these solutions for soil virus extraction efficiency. For agricultural soils, 250 mmol/L potassium citrate solution shows the highest extraction efficiency, though the resulting solution is viscous and difficult to filter or process for fluorescence microscopy counting. Sodium pyrophosphate is slightly less efficient but allows direct counting via fluorescence microscopy. Ten mmol/L potassium citrate solution is most suitable for extracting various phages from sludge. The choice of extraction solution depends on the target virus type and soil physicochemical properties.

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2. Separation, Purification, and Concentration of Soil Virus Solutions

2.1 Tangential Flow Filtration (TFF)

Tangential flow filtration is a method where liquid flows perpendicular to the filtration direction. Compared to conventional filtration, TFF generates shear forces on the filter surface that reduce particle accumulation, ensuring stable filtration rates. TFF is primarily used to separate and concentrate viral particles from large environmental samples. The system uses a hollow fiber membrane filter column; for example, a 0.2 m microfiltration column removes bacteria and protozoa larger than 0.2 m, while allowing smaller viruses to pass. The filtrate is then concentrated using a 100 kDa ultrafiltration column, where viruses are retained and returned to the retentate while aqueous solution passes through. Through repeated recirculation, the virus solution volume is reduced to less than 100 mL, yielding a relatively pure concentrate suitable for downstream analysis.

[Figure 2: see original paper] TFF filtration system and procedure[13]

2.2 Polyethylene Glycol (PEG) Precipitation

PEG precipitation is commonly used for virus purification and concentration. The method works by disrupting the hydration layer on viral capsid proteins, causing precipitation. To concentrate viruses, PEG 6000 is added to a final concentration of 10% and incubated overnight at 4°C. After centrifugation at 8000 r/min for 30 minutes, the viral pellet is resuspended in 1-2 mL of buffer for subsequent gradient centrifugation or density gradient purification.

2.3 Density Gradient Centrifugation

Since viruses vary in size and density, density gradient centrifugation can be used for both concentration and purification. Each virus type has a characteristic density that corresponds to a specific medium layer. This method is primarily suitable for pure virus separation, though it has the disadvantage of requiring expensive equipment not commonly available in standard laboratories[13].

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3. Quantification Techniques

3.1 Fluorescence Microscopy (Epifluorescence Microscopy, EFM)

The most commonly used method for virus quantification is epifluorescence microscopy. Diluted virus solutions are filtered onto membranes, stained with fluorescent dyes such as Yo-Pro-1, SYBR Green I, or SYBR Gold, and visualized under fluorescence. Viral particles are counted using an ocular grid and extrapolated to calculate the number of viruses per gram of soil. Compared to transmission electron microscopy, this method is simpler, more reproducible, and suitable for quantifying unknown viral particles in soil samples. However,

soil contains high levels of humic acids that can cause strong background fluorescence interference, potentially leading to overestimation of viral abundance as non-viral particles may also be stained[14,15].

3.2 Transmission Electron Microscopy (TEM) Classification and Counting

Before molecular biology techniques became available, TEM was the primary tool for viral ecology research. For soil samples, extracts are filtered and purified, then applied to Formvar-coated copper grids. After negative staining with 2% uranyl acetate for 1-2 minutes and removal of excess stain, samples are observed at 650× magnification for overview and then at higher magnifications for virus morphology and classification. This method is direct, accurate, and can visualize viruses that cannot be seen by fluorescence microscopy, though it requires sophisticated instrumentation.

3.3 Flow Cytometry (FCM)

Flow cytometry uses laser light to detect biological particles and enables multi-parameter rapid analysis and sorting. Samples are stained with fluorescent dyes and injected into a flow cell where sheath fluid forces cells or particles into single file for detection. This method is highly sensitive and provides the most accurate results for high-concentration viral samples, but cannot effectively detect low-concentration samples[16]. Like fluorescence microscopy, flow cytometry can only detect double-stranded DNA viruses that are fluorescently labeled.

3.4 Virus Counter

The virus counter is a specialized flow cytometer redesigned for viral particle detection. It uses a dual-staining system to label both viral genomes and envelope proteins, producing yellow and red fluorescence signals respectively. Only particles producing both signals are counted as intact viruses. Quantification can be completed in 5 minutes and can be automated. This method offers faster analysis, simpler sample preparation, and higher sensitivity than plaque assays, but is limited to detecting human and animal viruses and cannot represent environmental viral communities due to high instrument costs.

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4. Molecular Biology Methods

4.1 Nucleic Acid Extraction

Viral nucleic acids are extracted from concentrated viral solutions using DNase I to degrade free DNA before lysis. Various kits are available, including Mobio' s PowerViral Environmental RNA/DNA Isolation Kit, Qiagen' s QIAamp Viral RNA Mini Kit, and phenol-chloroform extraction combined with formamide[13,17]. Different soil types require different methods with

varying extraction efficiencies. If concentrations are low, Phi29 DNA polymerase amplification can be used. For RNA viruses, reverse transcription is required before downstream analysis. The most critical aspect is preventing contamination from other microorganisms and 16S rRNA.

4.2 Target Gene Diversity

A major reason viral ecology lags behind bacterial and eukaryotic research is the lack of universal marker genes among viruses. However, scientists have identified relatively conserved marker genes for specific viral groups, including capsid protein genes, capsid assembly protein genes, auxiliary metabolic genes, and various polymerase genes. Commonly studied markers include the g20 gene encoding capsid assembly protein in T4-type phages, which has been widely used in marine and freshwater environments[20-24]. Other markers include the mcp gene for major capsid protein[30-33], the phoH gene related to phosphate starvation[34-35], and DNA polymerase genes such as polA in T7-type phages and polB in Podoviridae[36-39]. These signature genes enable assessment of specific viral diversity in environmental samples.

4.3 Fingerprinting Techniques

With rapid molecular biology development, fingerprinting techniques like pulsed-field gel electrophoresis (PFGE) have been introduced to study phage community composition at the genome level. PFGE can separate viral genomes ranging from 45–125 kb, revealing diversity and enabling comparative analysis of phage distribution across environments. Studies of marine cyanophages have shown that while most isolates share morphological similarities with siphophages, their genome sizes vary widely. Research on vibriophages indicates that abundance correlates more with habitat type than geographic location[4].

4.4 Metaviromics Sequencing

Metaviromics is a high-throughput sequencing approach to study viral diversity in environmental samples. Current methods include traditional Sanger shotgun sequencing and next-generation sequencing (NGS) platforms: Roche 454 GS FLX, ABI SOLiD, and Illumina Solexa. The process involves extracting viral nucleic acids, adding adapters to both ends, quality control, and attaching fragments to beads or chips to create millions of micro-reaction pools. Amplified copies serve as sequencing templates[44]. After sequencing, the greatest challenge is data analysis. Raw reads undergo quality control and optimization, followed by gene prediction using tools like MetaGene. Sequence assembly and annotation against databases (KEGG, COG, SEED, ACLAME) provide functional and abundance information. However, a large proportion of viral sequences remain unknown, and annotation is difficult due to limited reference databases.

Compared to traditional methods like electron microscopy or targeted gene se-

quencing, metaviromics is the only approach that can comprehensively reflect viral community diversity and function[45]. It overcomes the lack of universal marker genes and can be used for phylogenetic analysis[46]. However, current soil viral metagenomic studies predominantly focus on double-stranded DNA viruses, with few reports on single-stranded DNA (ssDNA) viruses, likely due to low genomic content and extraction difficulties. The predominance of ssDNA viruses in some soils may result from amplification bias of Phi29 polymerase, which selectively amplifies circular genomes[48].

Dominant viruses in different soils and research methods

Soil Type	Typical Viruses	Genome Type	Method	References
Delaware soils	Caudovirales (tailed phages)	dsDNA	Viral clone libraries	[49]
Desert/Prairie	Actinophages (ΦAsp2)	dsDNA	Metaviromics (MDAH)	[1]
Rainforest soil	Myoviridae, Siphoviridae (-like)	dsDNA	Metaviromics (MDAX)	[50]
Paddy soil	Microviridae, Circoviridae	ssDNA	Metaviromics	[48]
Surface soil	Siphoviridae	dsDNA	Metaviromics	[51]
Hypolith (Namib desert)	Siphoviridae	dsDNA	Metaviromics	[17]
Machair (coastal sand)	Siphoviridae	dsDNA	Metaviromics	[52]
Brown earth (Scotland)	Microviridae	ssDNA	Metaviromics	[52]
Antarctic soil	Microviridae	ssDNA	Metaviromics	[51]

MDAH: DNA polymerase with heat treatment; MDAX: DNA polymerase without heat treatment

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5. Research Outlook

Metaviromics is undoubtedly the most comprehensive method in viral ecology, generating the largest datasets. However, analyzing only viral genomic information is insufficient. Future research must develop better bioinformatics tools and software to mine viral diversity and its relationships with environmental factors. Mechanisms of viral-mediated horizontal gene transfer and transposon

activity require further investigation, as do interactions between microbial and viral genomes.

Methodological development will drive future progress. Current studies focus primarily on double-stranded DNA viruses, but targeted research on soil viral communities should expand. Establishing standardized technical systems and soil virus resource banks will facilitate the development of beneficial viruses and control of harmful ones. Integrating bioinformatics, mathematical modeling, and interdisciplinary approaches will be crucial for advancing viral population dynamics and metaviromics data analysis.

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