*Minireview*

# Revolutionized virome research using systems microbiology approaches

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### Impact Statement

To address the virus population, virome is a viral metagenomic approach that can be utilized for viral surveillance in both research and clinical practice, with some potential advantageous applications over conventional strategies, especially concerning the information of the whole viral entity in the community. In this review, we describe the viral metagenomic approach to investigate the virus community and interactions with other microbial members as well as their hosts. We also summarize challenges, limitations, and benefits of the current virome approaches along with the potential applications of the viral metagenomic. Therefore, this review provides fundamental knowledge of using a virome approach for both research and clinical practice.

### Abstract

Currently, both pathogenic and commensal viruses are continuously being discovered and acknowledged as ubiquitous components of microbial communities. The advancements of systems microbiological approaches have changed the face of virome research. Here, we focus on viral metagenomic approach to study virus community and their interactions with other microbial members as well as their hosts. This review also summarizes challenges, limitations, and benefits of the current virome approaches. Potentially, the studies of virome can be further applied in various biological and clinical fields.

**Keywords:** Virome, systems microbiology, virus discovery, virus–host interaction, viral metagenomic

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## Introduction

Recently, the pandemic of COVID-19 has become a major world problem, emphasizing the importance of virus research. Heretofore, many viruses have been discovered either by laboratory research or by etiological identification in patients; however, huge numbers of viruses remain undiscovered.[1,2] Systems microbiology is a multidisciplinary area of microbiology, emphasizing both whole microbiome entities and microbial impact on their hosts or other microbes.[3,4]

Microbiomes refer to microbial communities in specific areas, and the microbes consist of archaea, bacteria, small eukaryotes, fungi, and viruses.[5] Previously, microbiome research has addressed the bacterial profiling and their relationships to the hosts, while virome studies are small in number. These virome studies have focused on both viral composition in the community and influence of the viruses (i.e., prokaryotic viruses, eukaryotic viruses, and endogenous viruses) on their hosts.[6,7] From the clinical perspective, many diseases have been identified as viral infections, and many are unknown; however, most virus infections have no effective treatment or specific drug for treatments.[8–10] In future, virome research will play an important role as a resource of knowledge for clinical applications, innovation of novel therapy, and prediction of virus outbreak patterns and of evolutionary processes of viruses in nature.[11,12]

In this review, we would like to discuss the revolutionization of virome research, including a summary of recent databases and bioinformatic analysis tools, viral interactions to their hosts and other microbes, and potential applications by the integration of high-throughput metagenomic technologies and systems microbiology approaches.

## The viral community revealed

Viruses are the most biologically diverse and biomassive entity on Earth. Currently, 5560 virus species across 150 families have been defined by the International Committee

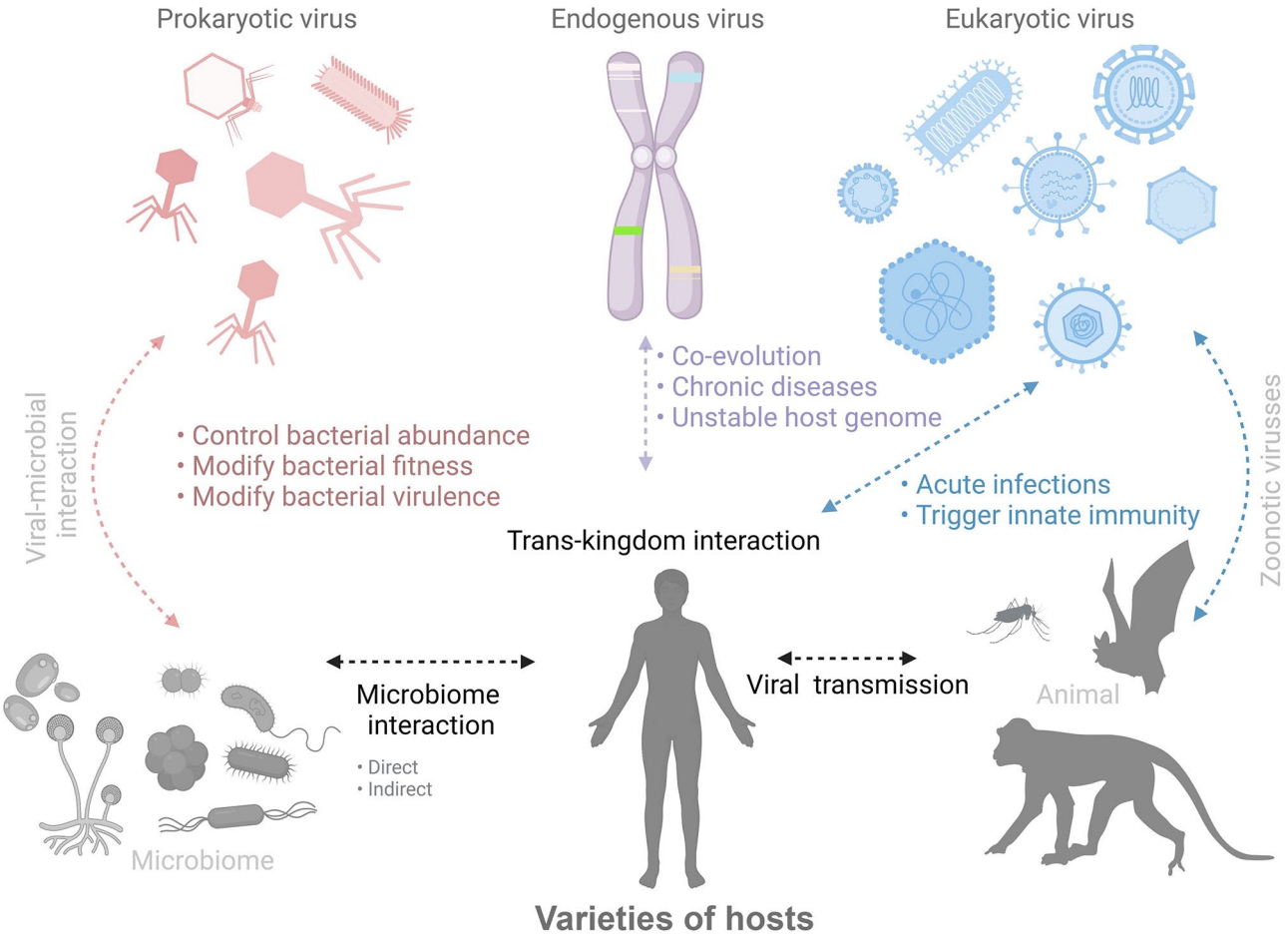


**Figure 1.** Overview of trans-kingdom interaction among viromes and hosts community. Figure created using BioRender (https://biorender.com/). (A color version of this figure is available in the online journal.)

on Taxonomy of Viruses (ICTV). The virus entities have diverse structures in terms of sizes and shapes, ranging from roughly spherical to linear, to amorphic. Furthermore, viral genetic materials are, both chemically and topologically, extremely diverse, consisting of single- or double-stranded DNA or RNA, covalently linked to proteins or chemically modified termini, and existing as a single molecule linear, separate segment linear, or circular forms.[8,9,13] Hence, this area of studies has focused on all types of viruses including bacteriophages (prokaryotic-infection viruses), endogenous viruses (viral elements, virus-derived genetic elements, prophages, and endogenous retroviruses in host genomes), and eukaryotic viruses (eukaryotic-infection viruses), as shown in Figure 1.

The vast majorities of viruses present on earth are lysogenic (with a latent period in host cells known as prophase) and lytic (infecting and killing the host shortly) phages which influence bacterial communities. With viral metagenomic approaches, bacteriophages were found to represent a large proportion of the microbial community.[11] Indeed, examinations of the human gut virome have revealed that crAssphage and its relatives are the predominant viruses in the gut viral community.[14]

The endogenous viruses, such as adenoviruses, herpesviruses, polyomaviruses, and circoviruses, provide invaluable information of ancient viral infections by integrations of the virus genome to their hosts. Generally, these viruses have been revealed to be associated with mammalian evolution (i.e., biological functions, emerging of placentation, immune adaptation and modulation, oncogenesis, and disease progression).[15,16] Moreover, approximately 8% of the human genome contains endogenous virus genes, related to many diseases including cancers, amyotrophic lateral sclerosis, multiple sclerosis, and rheumatoid arthritis.[17–19]

Many infectious diseases in both humans and animals are caused by eukaryotic viruses (e.g., influenza viruses, rotavirus, arboviruses, hepatitis C virus, and HIV). Indeed, these viruses (>60%) have been revealed to possess the capacity of transmission across host species (zoonotic transmission), leading to major problems around the world (i.e., pandemic, economic loss, and human health issues).[20,21] Therefore, the virus metagenomic approaches have been emphasized as novel strategies of virus surveillance, providing information of the entire viral community and having a potential for further applications to be discussed below.[22,23]

## Viral metagenomics

The development of high-throughput next-generation sequencing (NGS) provides both insights and possibility

in the accurate and timely detection and sequencing of nearly the full genome of several viruses in both clinical and research areas.[11,12,24,25] Fundamentally, there are five main processes for virome research workflow, including (i) sample collection, (ii) sample processing, (iii) sequencing, (iv) bioinformatic analysis, and (v) post-processing, as shown in Figure 2.[26–29]

(i) ***Sample collection.*** One of the most important steps is sample collection, since the accuracy and reliability of the analysis outcome can be significantly influenced by this process. Indeed, the main objectives are to preserve specimens (i.e., viral nucleic acids and community integrity) and minimize possible confounding factors for further downstream processes. To these aims, the recommended and considered condition as gold standard for long-term storage is −80°C. Alternatively, preservative reagents may be required for the samples from remote areas (i.e., rural or forest) until access to a −80°C freezer is provided.[30,31] In addition to storage temperature, freeze–thaw cycles also impact on the analysis outcome, such as virus and other microbe reads, by reducing the viral nucleic acid integrity in certain viruses.[32–34]

(ii) ***Sample processing.*** Typically, the aim of this process is to minimize host and environmental burdens (i.e., host genomes and cellular debris) and to maximize viral genome as much as possible for the requirement of the sequencing process to be fulfilled.[25,35] Of note, the filtration strategies can be carried out by passing the sample through porous filters; however, the size of targeted viruses is of concern. This is because, most often and commercially, the pore size of the filters is 0.45, 0.22, or 0.1 µm, so that large viruses (i.e., giant viruses and vaccinia viruses) may be retained in the filters, reducing the recovery yield, leading to underrepresentation of the viruses.[25,36] In this case, pretreatment of the filters with appropriate reagents (i.e., buffers, 10% fetal calf serum, or veal infusion broth) may alleviate the retention of the viruses.[37] A nuclease treatment may also be required for high level of host and bacterial genome contaminations before the viral nucleic acid extraction process.[38,39] For instance, samples for study of a whole bacteriophage community are usually contaminated with bacterial host genomes which may affect the downstream analysis due to the phages sharing genes with homology to bacterial genes.[40–42] Once the viral particles have been enriched, the appropriate viral genome extraction is required (i.e., formamide, thermal shock, or Phenol:Chloroform:Isoamyl alcohol extraction procedures, depending upon sample types). To enrich the viral genome, retro-transcription and/or amplification strategies, such as SISPA, RP-SISPA, LASL, and MDA, may be carried out before the library preparation.[43–46] Alternatively, the recent strategy of probe-capture-based technique for specific viruses (i.e., VirCapSeq-VERT for vertebrate viruses and Twist Respiratory Virus Research Panel

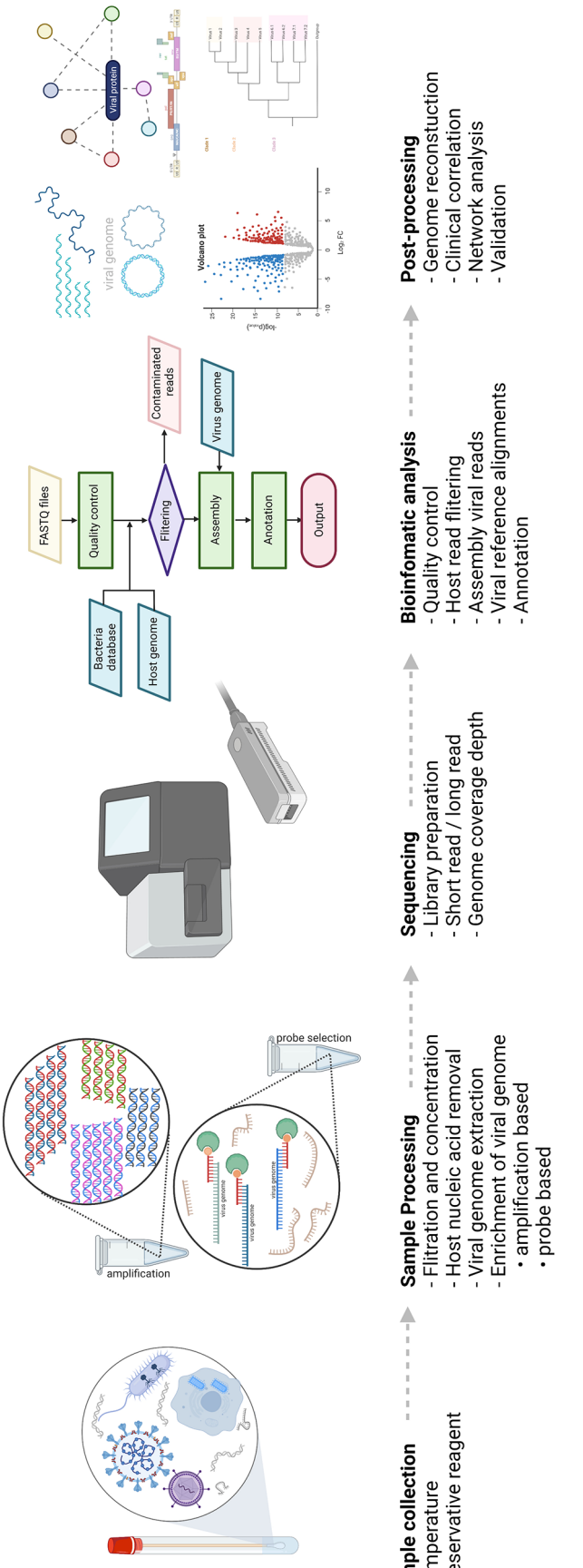


**Figure 2.** The workflow of virome research based on metagenomic technique. Figure created using BioRender (https://biorender.com/). (A color version of this figure is available in the online journal.)

for respiratory viruses) may helpfully enrich the targeted viral genomes.[47,48]

(iii) ***Sequencing.*** For virome metagenomic analysis, the sequencing platforms should be carefully selected and conducted, depending on sample types, viral genome yield for minimum concentration requirement of library preparation, the objective of the study, and the influence of the platforms on the number of viral reads and coverages.[49,50] In recent years, long-read sequencing platforms, such as Pacific Biosciences (PacBio) and Oxford Nanopore Technologies (ONT), have gradually increased interest in virome research on account of the advantages in the long-read datasets (i.e., detection of methylated sequences within viral genomes and high power of viral discovery for novel viruses).[51–53]

(iv) ***Bioinformatic analysis.*** In microbiome research, bioinformatic analysis pipelines for virome analysis essentially involve the non-viral genome removals based on *in silico* approaches to reduce computational resources and time-consuming analysis processes.[54] Indeed, there are many bioinformatic tools with different objectives for specific virome research. Moreover, they have shared common procedures, including *de novo* assembly before being aligned with reference genomes, as well as representative protein annotation from the virus sequences.[55] This review summarizes both commonly used bioinformatic pipelines and tools for virus identification by viral genome alignment and/or assembly, including the detection of all viruses, bacteriophages, and endogenous viruses with integration sites (Table 1), and viral reference databases with their main applications in virome research (Table 2).

(v) ***Post-processing.*** Once viral sequencing data sets have been completely analyzed in the bioinformatic pipeline, this process usually involves data interpretation and validation.[55,56] Typically, the assembled viral genomes will be investigated for their genome coverage. In case of poor viral genome constructions, the specific set of primers will be applied for the virus genomes that will be resequenced in order to obtain their complete genomes before other downstream processes.[57] As for novel viruses, validation strategies (i.e., RT-PCR and Sanger sequencing) are usually performed to ensure the findings. In addition, viral communities in the samples such as clinical specimens may often be required in the clinical interpretations. This is through visualization and statistical processes (i.e., heat map, phylogenetic construction, virome profiling, and network analysis) to obtain an insight into the viral community and the specimens.[11,16,38]

## Trans-kingdom interaction

The advancements in sequencing technology have provided the possibility of elucidating the microbial community. To understand how biological events are associated with the clinical outcome/manifestation of the diseases, the studies of viral interactions between other microbes (i.e., bacteria and fungi) as well as viral–host interactions by using combinatorial approaches (i.e., conventional cell biology and NGS technology) can provide the insight of both direct and indirect relationships among trans-kingdom interactions.

### Virus–microbe interaction

To this point, numerous studies have investigated the trans-kingdom relationship between microbes living inside human and animal bodies, especially the viral–bacterial relationship. Fundamentally and essentially, viruses have to bind to bacteria or bacterial products for initiating interactions. The viral–bacterial interactions can be simply classified into two major categories: direct and indirect interaction.[11,84,85]

For direct interaction, viruses and bacteria directly bind to each other for mutual benefit. Previously, several studies have revealed that the bacteria localized within host organisms promote the viral infections and vice versa.[86,87] Indeed, the respiratory bacterial pathogens, such as *Streptococcus pneumoniae*, *Moraxella catarrhalis*, and *Staphylococcus aureus*, have an enhanced adherence capacity to the host epithelial cells when these microbial surfaces are bound with influenza virus, promoting the secondary bacterial infection. Moreover, viral co-infection of different enteric viral strains can be enhanced by the bacterial mediation, and the viral fitness can also be increased by microbes through genetic recombination events of these viruses, reducing the deleterious mutations.[88–90]

The other benefit of the interaction among viruses and bacteria is virion stability. In this case, bacteria provide the microenvironment of their cell walls (i.e., lipopolysaccharide, peptidoglycan, lipoteichoic acid, and *N*-acetylglucosamine-containing polysaccharides) to the viruses to enhance the stability of the virion during transmission or environmental exposure.[90–93] For example, virion thermal stability of poliovirus, coxsackievirus, human norovirus, and reovirus is promoted in the presence of certain bacteria, while Aichi virus and mengovirus have been discovered to be more vulnerable. Moreover, the interaction among bacteria and viruses has been shown by the increased capacity of bleach.[90,93] These studies have revealed, and provided clues concerning, the enhancement of the chance and duration of their active progeny to survive during environmental exposure before binding to their target host cells. From this interaction, it has been intriguing to establish further investigation of the wider aspects of the virome–bacteriome association, to gain more knowledge and understanding of the viral–bacterial relationship.

Furthermore, the viruses and bacteria can be indirectly correlated through bacterial production. In the case of human noroviruses and commensal enteric bacteria, the histo-blood group antigen (HBGA) expressing bacteria may promote the viral infection to the human B cells by some unknown mechanisms.[94] The biotransformations of bacteria are prominent in the gut microbiome. In fact, bile acids can be transformed by gut microbes to secondary bile acids and other products.[95] In the presence of certain bile acids, the

**Table 1.** Selected bioinformatic tools and pipeline for virome analysis.

| Tools | Description | Strength | Year | References |
|---|---|---|---|---|
| Viruses | | | | |
| METAVIRALSPADES | Identification of viral genomes by using a set of virus-specific hidden Markov models for metagenomic assembly analyzing with variations and coverage depth | • Neural network analysis<br>• Novel virus discovery in diverse metagenomic datasets | 2020 | Antipov *et al.*[58] |
| virMine | Identification of viral genomes from raw reads representative of viral or mixed (viral and bacterial) communities using an iterative approach for read quality control, assembly, and annotation | • Alternative mode between specific study system and/or feature(s) of interest<br>• Novel species detection | 2019 | Garretto *et al.*[59] |
| Kraken 2 | Classification and assigning taxonomy of metagenomic sequences with BLAST program in the fastest mode | • Low memory usage<br>• High speed<br>• High sensitivity | 2019 | Wood *et al.*[60] |
| FastViromeExplorer | Detection and abundance quantification of viruses and phages in large datasets by performing rapid searches with pseudo-alignment tool for RNA-seq data | • RNA-seq data analysis<br>• Rapid mapping of short metagenome reads<br>• Suitable for limited computing power research | 2018 | Tithi *et al.*[61] |
| VirMAP | Combination of nucleotide and protein metagenomic datasets for taxonomic classification of viral genome reconstructions | • Combinatorial analysis of nucleotide and protein sequences<br>• Virus surveillance capabilities | 2018 | Ajami *et al.*[62] |
| EZ-Map | Metagenomic analysis of human virome with python-based tools for filtering, alignment, and analysis from cell-free DNA data sets | • Fully automated computational pipeline for both workstations and computing clusters<br>• Suitable for cell-free DNA datasets | 2017 | Czeczko *et al.*[63] |
| VirusDetect | Using small RNA sequences strategy with homology of reference-alignment and *de novo* assembly | • Small RNA sequence analysis<br>• Potential novel virus identification<br>• Highly sensitive and efficient identification | 2017 | Zheng *et al.*[64] |
| VirFinder | Identification of viruses by using machine learning with k-mer based approach for mixed metagenomes containing both viral and host sequences | • A web-based tool<br>• An alignment-free tool using machine learning<br>• High potential to detect novel virus | 2017 | Ren *et al.*[65] |
| VirusSeeker | BLAST-based NGS data analysis pipeline for both novel virus discovery and virome composition analyses | • False-positive removal<br>• Detection of both RNA and DNA viruses in different families. | 2017 | Zhao *et al.*[66] |
| Bacteriophage | | | | |
| VIBRANT | The hybrid tool using machine-learning and protein-similarity approach for recovery and annotation of viruses and microbes with the curation of predictions, estimation of genome quality, and infection mechanism | • Low false positive<br>• Discovery of phage–microbe interactions | 2020 | Kieft *et al.*[67] |
| PPR-Meta | Identification of both phage and plasmid fragments from metagenomic using Bi-path convolutional neural network | • Available for a local PC<br>• Identification of phages and plasmids<br>• Novel phage identification | 2019 | Fang *et al.*[68] |
| MARVEL | Using a random forest machine-learning approach for prediction of double-stranded DNA bacteriophage sequences in metagenomic bins | • High sensitivity<br>• Novel phage identification | 2018 | Amgarten *et al.*[69] |
| PHASTER | Phage search tool for identifying and annotating prophage sequences within bacterial genomes and plasmids | • Web-based tool<br>• Identification and annotation of prophage sequences | 2016 | Arndt *et al.*[70] |
| Endogenous virus | | | | |
| DeepVISP | Viral integration site prediction using convolutional 6 neural network (CNN) models in the human genome | • Online tool server<br>• Accurate prediction of oncogenic virus integration sites<br>• Identification of biological or regulatory roles with unknown integration site | 2021 | Ren *et al.*[71] |
| detectedIS | Identification of exogenous DNA integration sites in a plasmid containing transgenes or virus sequences based on a Nextflow workflow combined with a singularity | • Able to use DNA or RNA paired-end sequencing datasets<br>• Accurate and lower computational demand with less execution times. | 2021 | Grassi *et al.*[72] |
| SurVirus | Viral integration caller with alignment correction of reads for the discovery of integrated sites | • Detection of novel virus integration site with less noise<br>• Quick scan large data sets | 2021 | Rajaby *et al.*[73] |
| VIcaller | Identification of viral integration events using high-throughput sequencing (HTS) from human dataset through virome-wide screening of clonal integrations under Linux platform. | • Identification of breakpoint of viral integrations in human genome caused cancers<br>• Compatible with whole genome and RNA-seq datasets | 2019 | Chen *et al.*[74] |
| Seeksv | Detection of somatic structural variants and viral integration using different types of sequencing data | • High efficiency and precision<br>• Identification of breakpoint located in sequence homology regions | 2017 | Liang *et al.*[75] |

**Table 2.** Database of virome.

| Database | Main applications | Source link | References |
|---|---|---|---|
| EBI | European Nucleotide Archive<br>• Viral reference sequences<br>• Viral taxonomy | https://www.ebi.ac.uk/genomes/virus.html | – |
| HVPC | The human virome protein cluster<br>• Human viral protein database<br>• Diversity<br>• Functional annotation | https://osf.io/gs4zf/ | Elbehery *et al.*[76] |
| IMG/VR | Integrated microbial genome/virus<br>• Cultured and uncultured DNA/RNA viral genome sequences<br>• Integrated ecological and evolutionary database | https://img.jgi.doe.gov/cgi-bin/vr/main.cgi | Paez-Espino *et al.*[77] |
| MVP | Microbe versus phage<br>• A microbe–phage interaction database | http://mvp.medgenius.info/home | Gao *et al.*[78] |
| NCBI virus | National Center for Biotechnology Information Virus<br>• Viral reference sequences<br>• Viral taxonomy | https://www.ncbi.nlm.nih.gov/labs/virus/vssi/ | Hatcher *et al.*[79] |
| PhagesDB | Actinobacteriophage database<br>• Genomics of phages<br>• Bacterial hosts<br>• Viral taxonomy | https://phagesdb.org/ | Kaján *et al.*[15] |
| pVOGs | Prokaryotic Virus Orthologous Groups<br>• Complete set of orthologous gene families<br>• Multiple complete genomes of bacterial or archaeal viruses | http://dmk-brain.ecn.uiowa.edu/pVOGs | Grazziotin *et al.*[80] |
| ViPR | Virus Pathogen Resource<br>• Viral reference sequences<br>• Gene and protein annotations<br>• Immune epitopes<br>• 3D structures<br>• Host factor data | https://www.viprbrc.org/brc/home.spg?decorator=vipr | Pickett *et al.*[81] |
| ViralZone | Virus knowledge resource<br>• A comprehensive resource of viral genomic and proteomic sequences<br>• Reference strains<br>• Virion pictures | https://viralzone.expasy.org/ | Hulo *et al.*[82] |
| Virus–Host DB | Virus–Host Database<br>• Relationship of viruses and hosts<br>• Taxonomy viruses and their hosts<br>• Complete genomes of reference sequences and host sequences | https://www.genome.jp/virushostdb/ | Mihara *et al.*[7] |
| ViruSurf | ViruSurf: an integrated database<br>• Integrated and curated metadata and viral sequences from heterogeneous sources<br>• Analytical dimension characterizes the sequence | http://gmql.eu/virusurf/ | Canakoglu *et al.*[83] |

receptor binding protruding domain of noroviruses is stabilized and the binding capacity to the host cells is enhanced through corrected conformational rotation of the domain by the electrostabilizing effect between the viral domain and the bile acids.[96,97]

Although the conventional biological approaches have offered an insight into the viral–bacterial interactions by focusing on a part of the community, the residual microbiota (i.e., bacteriophages, fungi, and other bacteria and viruses) should also be investigated to complete our knowledge of trans-kingdom relationships. Metagenomic approaches to the viral studies provide a different point of view from those of conventional approaches in the focus on the whole community.[38,98–100] Nonetheless, in most of the virome studies focusing on the viral–bacterial interaction, the viruses and other microbial relationships (i.e., viral–fungal interaction) have not yet been well studied. Thus, future investigations of virome studies should be established in different aspects of the community, in order to reveal the uncovered parts of the microbiota relationships, and ultimately lead to novel therapeutic strategies.

## Virus–host interaction

Through the virome metagenomic approach, several studies have revealed that there are numerous viruses residing in/on the human body, such as skin, oral cavity, lung, gastrointestinal tract, and blood, with both commensal and pathogenic associations with their hosts.[11,85]

With respect to virus–host interactions, previous studies have shown that host behavior and geographic locations have influences on viruses. The dietary factor is an obvious example, having an effect on viral community structure. The viral infections of infant guts and mortality from viral gastroenteritis are lowered in infants who are received breastfeeding.[101–103] In fact, maternal breast milk is composed of many components (i.e., maternal antibodies, oligosaccharides, and lactoferrin) which influence the viral community,

especially Adenoviridae, Picornaviridae, Parvoviridae, and Caliciviridae in the early life.[11,101,102,104,105] Furthermore, a recent study has demonstrated that food types (i.e., staple foods, side dishes, fruits, and beverages) have been correlated to the virome structure in the human body.[106]

In addition to the host's behavior, host geography is one of the main factors affecting virome structure.[106–109] A comparative study of diarrhea-related viruses has shown that Picornaviridae and Adenoviridae are significantly different between 2 different locations in Australia.[109] Moreover, a study of geography affecting virome structure among Chinese cohorts has revealed that viral communities among the cohorts are significantly distinct in the manner of virome diversity, evenness, and richness.[106,108] These may indicate that the hosts' behavior and geography are quite prominent effects on virome structure and could be categorized as a form of interaction between viruses and their hosts.

For some instances, eukaryotic viruses may provide benefits to their hosts. In gnotobiotic mice, murine norovirus (MNV) has been shown to have a beneficial function of compensating for commensal bacterial depletion. Of note, the virus also restored the intestinal morphology and lymphocyte function without any obviously adverse effects.[110] Likewise, a form of viral–host interaction has been discovered between astrovirus and the immunodeficient mouse host, in which the hosts were protected from MNV and rotavirus infection via inducing type III interferon inside the guts.[111]

Bacteriophages may indirectly interact with eukaryotic hosts through modification of bacterial composition and genetics or stimulation of host immune responses.[112] One study of Crohn's disease, for example, has shown the potential of phage therapy in reducing adherent invasive *Escherichia coli*.[113] In addition, phages may provide the reservoir of mobilizing genetic elements to their bacterial hosts for antimicrobial resistance.[43,114,115] This may promote the severity and mortality in some cases through indirect interaction between eukaryotic hosts and bacteriophages. For eukaryotic host immunity, bacteriophages could stimulate the host immune response, without bacterial mediation, through Toll-like receptor (TLR) signaling, in which type I interferon is induced by the phages as well as the production of IL-6, IL-10, IL-12, and IFN$\gamma$.[116,117]

## Challenges and limitations

Viruses are the most diverse on Earth with the total number of virus-like particles estimated to be $10^{31}$ but only 1% have been discovered.[118] The advent of high-throughput sequencing technology, particularly metagenomic technologies, allows researchers to access the complexities of microbial communities including bacteriome, mycobiome, and virome. Although more advances of the technologies continually come forward, the virome studies still have their own challenges and limitations related to their sample and downstream analysis processes.[27,56,119] The major obstacles include comprehensively defining viral dark matter in the virome data set, and virome–host interaction studies, particularly virome studies in animal model experiments, are still limited for many reasons as described below.

### The viral dark matter

Viral dark matter is defined as the nucleotide sequence which originates from viruses that cannot align with any reference nucleotide or amino acid sequence.[120] In previous virome research that uses a purifying viral particles methodology, almost 40% to 90% of sequence were unalienable.[121–123] This challenge has been limited for a number of reasons that will be described in the two primary procedures (sample processing and bioinformatics) approaches.

*Sample processing.* In recent years, many attempts have been efficaciously applied to decrease the amount of viral dark matter. Even though there is no standard protocol for identifying all viruses, specific methods are assigned by specific attributes of a subgroup. Thus, these specific methods are significant when defining all compositions of the virome diversity.

For example, most clinical samples usually suffer from a low abundance of viruses along with a high background of their hosts or other microbes.[24,124] Using a filter of 0.22 μm may remove host cellular or bacterial burdens from the viral entities; however, this strategy can also deplete large viruses and reduce the amount of recovered viral DNA by half, establishing biases toward the most abundant of the viral community members.[125] Likewise, some phages have atypical buoyancy; thus, the CsCl gradient ultracentrifugation may promote the bias to specific phage types.[33,41] As with other techniques, this method still has some drawbacks including reducing the Virus-like particles (VLPs) and decreasing the sensitivity of viral detection.[126] Furthermore, most commercial genomics extraction kits focus only on either DNA or RNA. This may produce biases toward some type of viruses due to the viral community in virome research containing both DNA and RNA viruses.[127]

Although the virome provides culture-independent sequencing for most viruses without group-specific primers to differentiate the viral species like the 16s or ITS region in bacteria and fungi, respectively, the confounding noise from the background still impacts on sequencing datasets.[62] In addition, the metagenomic approach also recommends using the viral mock community aid in assessing biases into a virome pipeline. However, the viral mock communities comprise a limited number of reference materials to cover all types of different genetic materials.

*Bioinformatics approaches.* The next limitation in virus discovery is that none of the computational methods can always clearly identify viral sequences. Besides, some computational approaches limit the identification of novel viruses, such as nucleotide alignment.

However, to address the viral sequences, *de novo* assembly has been considered a major approach in this area.[42] As the complexity of the virome sequencing data increases (i.e., many repeat regions and genomic diversity), the assembly seems to be challenged in many studies, particularly in the validity of the viral annotation.[42] In some cases, during viral genome assembly, mismatches from synonymous single-nucleotide polymorphisms can arise due to limitations of the tools and databases used. One strategy to resolve this

issue is utilizing the viral protein amino acid sequences, as in Plass.[128] This use of viral protein amino acid sequences and/or machine-learning analysis has shown potential to predict novel viruses from the sequences and to improve function prediction.[42,128,129]

The database is a critical module for identifying viral sequences. Although the number of viruses in the database has been rapidly increased, the sequences are largely biased toward mammalian, plant, and bacterial viruses. There is still a small number of fungal protist and archaeal viruses in RefSeq GenBank.[79] In this regard, a number of selected bioinformatics tools and databases are summarized in Tables 1 and 2. Furthermore, the limits of identified viral diversity by Baltimore classification and host relations also possibly encourage the high incidence of viral dark matter.

### Unrevealed virome–host interaction studies

Although some virus–host interaction has been elucidated by combinatorial approaches to understand the biological events, two of the biggest remaining challenges are uncultured viruses and animal models.

*Uncultured viruses.* Most of the viruses discovered through metagenomic approaches are unculturable viruses. This raises the topic of discussion of these viruses in identification of their host ranges, especially bacteriophages. As aforementioned, bacteriophages may indirectly interact with eukaryotic hosts through modification of bacterial entities. Therefore, the determination of their bacterial host range may give an indication of their trans-kingdom relationship. Currently, the culture-independent approaches have been utilized for host range prediction such as viral tagging and *in silico* predictions.[130–133] For viral tagging, this technique utilizes fluorescence-activated cell sorting to separate the labeled phages, binding to their hosts, for further downstream analyses.[132–134] Alternatively, *in silico* predictions may apply the correlation among phages and their bacterial abundances as well as genetic signatures of phages (i.e., genetic homology between phages and bacteria, prophage integration, or CRISPR) to determine the host ranges.[35,131]

For eukaryotic viruses and bacteriophages, the lack of culture systems is a major limitation for virome research to accomplish Koch's postulations, the gold standard for microbial disease causality.[135–137] Indeed, previous studies have established primary airway epithelial cells to characterize the HCoV-HKU1 and human bocavirus.[138,139] To understand the nature of viruses in the gastrointestinal (GI) tract, the development of an organoid may be useful for investigation of the viruses and host interactions and may open the opportunity to establish a novel strategy of mock microbial communities.[36,140]

*Animal models.* It is well known that viruses are highly diverse and infect either eukaryotic or prokaryotic cells as obligate parasites. Thus, a specific host to establish specific types of animal models is necessary for virome research in this experiment. In order to manage the practical and ethical constraints, functional studies of the virome in humans should be limited, making animal models an invaluable experimental tool to understand its impact on physiology.[141]

Mice have been used as the robust models of virome-associated disorders. Thus, mice are often used as a proxy for human diseases to decipher their pathophysiology or test new therapeutics. Virome diversity correlates with several pathological conditions, such as inflammatory bowel disease, arthritis, and child growth impairment.[12,142,143] Macaques are also closely related to humans and are, therefore, a pertinent model to study parameters that influence the human virome. *Cynomolgus macaques* and *Rhesus macaques* have recently been used to analyze the effect of aging and chronic diarrhea on the virome, respectively.[144,145] Recently, rabbits have been used, leading to the identification of a novel polyomavirus.[146] Finally, swine models have also been used for virome in fecal virome transplants, which have been shown to act against necrotizing enterocolitis in pigs.[147]

However, the laboratory animals grow under specific pathogen-free (SPF) conditions, which means they have lower compositions of other viruses and other microbes compared to the natural animal.[148] Overall, the impact of the virome on most animal studies is still unclear; in particular, the phage physiological roles remain unclear in the host (human or animal) and bacterial communities. A further challenge lies in dissociating the effect of viruses from those of bacteria and between individual viruses, with the aim of identifying the mechanism of the virus in human health and disease.[141]

Moreover, in terms of data interpretation, one of the major challenges in virome research is determining the relationship between viruses and other factors in the community (i.e., age, genetic, health status, and/or diet of their hosts).[149,150] The multiple factor analysis (MFA) technique has been used in this area to examine the multivariate correlation between the viruses and their hosts.[151–153]

## Potential applications of virome research

Although there are many limitations in virome research, several applications of this field are continuously and increasingly being realized in both biological aspects and clinical areas. For biological applications, the virus surveillances using viral metagenomics have shown the possibility to expand virus databases, in which PCR- or panel-based techniques may be limited to the viruses.[38,154] Indeed, the virus databases might be utilized as reference genomes of both known and novel viruses as well as viral reference communities for standardized viral metagenomic approaches.

For clinical applications, viral metagenomics allows our understanding of host and microbiota relationships to be enhanced, which leads to possible alternative therapeutic strategies for several diseases. For example, oncolytic therapy based on viruses introduced into cancer cells (e.g., HSV, vaccinia virus, and adenovirus) has recently revealed potential modulations of the tumor microenvironment and cancer-related immunity in anticancer therapy.[155,156] Moreover, the efficacy of fecal microbiota transplantation has been demonstrated as a potential treatment for inflammatory bowel disease (IBD), *Clostridioides difficile* infection, severe colitis associated with graft-versus-host disease following hematopoietic stem cell transplantation, and obesity.[55,157–160] In addition, phage-based therapy might offer potential treatments

for IBD caused by *C. difficile* and *Escherichia coli*, as well as for colorectal cancer, in which the genes associated with cancer are down-regulated after being treated with *E. coli* bacteriophages. However, there are some issues of concern for the phage treatments, including the appropriate life cycle of phages for effective therapeutic effects, standardization of the protocol and legal frameworks for clinical application, and horizontal gene transfers across kingdoms.[64,113,161,162]

Diagnostically and prognostically, the virome approach may be applied to identify and reveal other potentially pathogenic viruses in clinical metagenomic fields, such as torque teno virus 7 for Kawasaki disease.[163] Furthermore, respiratory failure, periodontitis, and other illnesses have also been shown to have associations between viruses and symptoms using human virome approaches.[28,164] In this regard, the clinical applications of viral metagenomics in parallel with conventional practices may result in novel patient management and therapeutic strategies.[16]

Finally, virome research can also be used to address potential zoonotic viral transmissions. Indeed, the recent pandemic caused by SARS-CoV-2 may possibly come from zoonotic transmission, as several studies have demonstrated that viral transmission across species from bats (serving as original reservoirs) to pangolin which may have served as an intermediate host.[165] In addition, a study of fecal virome between wild and captive cynomolgus macaques has revealed three novel macaque viruses and illustrated the potential zoonotic transmissions of rabies and herpes B viruses to humans.[38,166,167] These insights could benefit our understanding and management of viral outbreaks in the future, especially in combination with viral metagenomic and artificial intelligence prediction.[168,169]

## Conclusions

In conclusion, the advent of metagenomics has changed the face of virome research through providing an insight into whole viral communities. Utilizing virome approaches, the interactions of both viruses and their hosts, as well as other microbial members in the community, can be accessed. Moreover, combinatorial analyses of virome and other systemic microbiology approaches (i.e., metabolomic, transcriptomic, culturomic, and proteomic) may allow us to establish models and interaction networks, used for specific purposes, such as pandemic preventions, regulation of virus populations, and research applications, based upon the roles of viruses in communities. However, there are many challenges and limitations in current techniques. Therefore, further inventions and investigations are essential for addressing the viral community and their relationships.

### AUTHORS' CONTRIBUTIONS

SC and SP contributed to the conception of this review article. SC and PS contributed writing and discussion of this review article. The final version of this article was approved by all authors.

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