Gut virome of mammals and birds reveals high genetic diversity of the family Microviridae

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Abstract

Nineteen families of phages infecting bacteria or archaea are currently recognized by the International Committee on Taxonomy of Viruses (ICTV). Of these, only two have single-stranded DNA genomes, namely Inoviridae and Microviridae. The distribution, genetic characteristics, and ecological roles of Microviridae remain largely under explored. Here, using viral metagenomics, we investigate the intestinal virome from human and twenty-four species of animals, as well as freshwater samples, containing abundant sequence reads showing similarity to the Microviridae. Eight hundred and sixty complete or near complete Microviridae-related genomes were generated, showing high levels of co-infections and sequence divergence. Sequence comparison and phylogenetic analysis showed that the Microviridae subfamily Gokushovirinae was highly prevalent and that some strains may qualify as new subfamilies. This study significantly augments our knowledge of the genetic diversity, genome evolution, and distribution in animal species of members of the family Microviridae.

Key words: Microviridae; complete genome; phylogenetic analysis; genetic diversity.

1. Introduction

Bacteriophages can influence microbial abundance, composition of microbial communities, and even biogeochemical cycling by interacting with their cellular hosts (Morella et al. 2018). A great deal of research has been performed with phages with double-stranded DNA (dsDNA) genomes (Salmond and Fineran 2015). Recent studies have revealed that members of the Microviridae family were found in water and the content of animal and human guts (Desnues et al. 2008; Tucker et al. 2011; Labonte and Suttle 2013; Hopkins et al. 2014; Bryson et al. 2015; Quaiser et al. 2015; Doore and Fane 2016; Guo et al. 2017; Barrientos-Somarribas et al. 2018; Xie et al. 2019), suggesting possible roles in affecting different environments. The properties of some members of the Microviridae family have been well elucidated particularly in virion structure and assembly (McKenna et al. 1992; Doore and Fane 2016; Blackburn et al. 2017). This family is currently comprised of two International
Committee on Taxonomy of Viruses (ICTV)-approved subfamilies: Bullavirinae and Gokushovirinae, and two tentative groups: Pichovirinae and Alpavirus, which have not been formally accepted by ICTV (Roux et al. 2012; Adams et al. 2016). The subfamily Bullavirinae includes three genera, Alpha3microvirus, G4microvirus, and Phix174microvirus, which are known to infect enterobacteria and have been extensively studied through the archetype of this family, the bacteriophage Phix174, while the Gokushovirinae subfamily is commonly referred to as ‘parasites of parasites’, which infect obligate intracellular parasites within the genera Chlamydia, Bdellovibrio, and Spiroplasma (Brentlinger et al. 2002).

Mammalian bodies support a diverse bacterial community residing in the lower gastrointestinal tract which itself hosts diverse phages. Using viral metagenomics, we investigated the virome in fecal samples from mammals and birds which revealed a great deal of divergent Microviridae sequences. The estimated abundance of these bacterial viruses in different animal species, genome organization, sequence similarity based on VP1 protein which is a classic phylogenetic marker for the classification of Microviridae (Hopkins et al. 2014; Quaiser et al. 2015), and phylogenetic diversity are analyzed here.

2. Materials and methods

2.1 Samples and preparation

During 2014–6, 1,795 samples, including 1,546 fecal samples from human and 24 species of animal, 80 cattle (Bos taurus) blood, 20 wild rat oral secretion, 27 cattle nasal secretion, 20 salmon secretion from human, 80 cattle genital secretions, 20 wild rat (Rattus norvegicus) skin swab, and 2 concentrated fresh water samples were collected from 13 different provinces in China (Fig.1a and Supplementary Table S1). All samples were collected by disposable materials and shipped on dry ice. These samples were collected as part of a Virome Project of China supported by the Ministry of Science and Technology of China aimed at the discovery of emerging viruses in mammals and birds. The samples obtained from humans were collected following informed written consent from each person.

Fecal samples were re-suspended in ten volumes of phosphate-buffered saline (PBS) and vigorously vortexed for 5 min. Fecal supernatants were then collected after centrifugation (10 min, 15,000 × g). The tips of swabs were immersed into 1 ml PBS and vigorously vortexed for 5 min and incubated for 30 min at 4 °C. The supernatants were then collected after centrifugation (10 min, 15,000 × g). Oyster (Ostrea gigas) digestive tissue samples were homogenized, frozen, and thawed three times on dry ice, the supernatants were then collected after centrifugation (10 min, 15,000 × g). The whole blood samples were centrifuged (10 min, 15,000 × g) for collection of plasma. Five hundred microliters of each supernatant was filtered through a 0.45-µm filter (Millipore) to remove eukaryotic and bacterial cell-sized particles. The filtrates enriched in viral particles were treated with DNase and RNase to digest unprotected nucleic acid at 37 °C for 60 min (Zhang et al. 2014, 2016; Liu et al. 2016).

2.2 Viral metagenomic analysis

Remaining total nucleic acid was then isolated using QIAamp MinElute Virus Spin Kit (Qiagen) according to manufacturer’s protocol. The enriched viral nucleic acid preparations of the respective pools or samples were individually subjected to reverse transcription reactions using reverse transcriptase (Super-Script III, Invitrogen) and 100 pmol of random hexamer primer, followed by a single round of DNA synthesis using Klenow fragment polymerase (New England BioLabs), where reverse transcription was included because of the original purpose of detecting DNA as well as RNA viruses.

Overall, 263 libraries were then constructed using Nextera XT DNA Sample Preparation Kit (Illumina) and sequenced using the MiSeq Illumina platform with 250 bases paired ends with dual barcoding for each individual sample or sample pool. The information about each library was shown in Supplementary Table S1. Sample collection and all experiments in the present study were performed with Ethical Approval given by Ethics Committee of Jiangsu University and the reference number is No. UJS2015022.

For bioinformatics analysis, paired-end reads of 250 bp generated by MiSeq were debarcoded using vendor software from Illumina. An in-house analysis pipeline running on a thirty-two-nodes Linux cluster was used to process the data. Reads were considered duplicates if bases 5–55 were identical and only one random copy of duplicates was kept. Clonal reads were removed and low sequencing quality tails were trimmed using Phred quality score ten as the threshold. The unique reads number of each library was shown in Supplementary Table S1. Adaptors were trimmed using the default parameters of VecScreen which is NCBI BLASTn with specialized parameters designed for adapter removal. The cleaned reads were de novo assembled within each barcode using the ENSEMBLE assembler (Deng et al. 2015). Contigs and unassembled reads are then matched against a customized viral proteome database using BLASTx with an E-value cutoff of <10−5, whereas the virus BLASTx database was compiled using NCBI virus reference proteome (ftp://ftp.ncbi.nih.gov/refseq/release/viral/) to which was added viral proteins sequences from NCBI nr fasta file (based on annotation taxonomy in Virus Kingdom). Candidate viral hits are then compared to an in-house non-virus-non-redundant (NVNR) protein database to remove false positive viral hits, where the NVNR database was compiled using non-viral protein sequences extracted from NCBI nr fasta file (based on annotation taxonomy excluding Virus Kingdom). Contigs without significant BLASTx similarity to viral proteome database are searched against viral protein families in vFam database (Skewes-Cox et al. 2014) using HMMER3 (Eddy 2009; Johnson, Eddy, and Pongualy 2010; Finn, Clements, and Eddy 2011) to detect remote viral protein similarities.

2.3 Acquisition of Microviridae genomes

For assembly of the Microviridae genomes, the contigs which showed significant BLASTx similarity to Microviridae were selected to check whether they are circular genomes in Geneious software version 11.0. Only those genomes that showed overlapping reads at the start and end of the contigs, confirming their circular genomes were retained. The contigs with sequence length >3,000 bp but without circular genome were subjected to further analysis where the individual contig was used as reference for mapping to the raw data of its original barcode using the Low Sensitivity/Fastest parameter in Geneious software version 11.0. The prolonged contigs were manually re-checked for determining whether they were circular genomes. Those contigs with sequence length >3,500 bp and the major capsid protein VP1 and the replication protein VP4 were considered to be nearly complete genomes and included in this study. The contigs which had sequence length >7,000 bp but have no putative protein VP1 or VP4 were considered to be non-Microviridae sequences and not included in this study.
2.4 Phylogenetic analysis

Phylogenetic analyses were performed based on the predicted amino acid of the major capsid protein VP1 of Microviridae and eighteen representative members of the previously confirmed subfamilies, Bullavirinae and Gokushovirinae, and the other six proposal subfamilies. Sequence alignment was performed using CLUSTAL W with the default settings. Phylogenetic trees with 1,000 bootstrap resamples of the alignment data sets were generated using the Maximum-likelihood method in MEGA7.0. For sequence pairwise comparison, the alignment data were imported into and analyzed by CLC Genomics Workbench 10.0, where the identities between sequences and the corresponding heat maps were generated. Potential viral open reading frames (ORFs) in Microviridae genomes were predicted by combining Geneious 8.1 software and NCBI ORF finder. The annotations of these ORFs were mainly based on comparisons to the Conserved Domain Database using RPS-BLAST with an E-value cutoff of <10^-5. For the coding protein sequences from ORFs which had no significant similarity found in Database were annotated as putative proteins.

2.5 Quality control

In order to exclude the possibility of contamination with viral nucleic acids present in the laboratory or from Qiagen nucleic acid extraction kits, twenty samples or pools positive for Microviridae whose best matches are related to marine gokushovirus or Eel River basin pequenovirus (Bryson et al. 2015; Creasy et al. 2018) were randomly selected and the nucleic acid were re-extracted using Trizol reagent (Invitrogen). PCR using primers specific to those 20 sequences confirmed their presence in the original biological samples.

3. Results

3.1 Sequence reads of Microviridae in different samples

The current study consisted of 1,796 samples, including intestinal content, blood, nasal secretion, genital secretion, and skin swab from human and 24 animal species plus two fresh water samples. All of these samples were pooled or individually used for library constructing and viral metagenomic analysis.
Overall, 263 nucleic acid libraries from these samples were generated and sequenced in 8 Illumina MiSeq runs. Sample and library information are shown in Fig. 1a and Supplementary Table S1. The viromes corresponding to the 263 libraries/barcodes were then generated, where sequences whose translated amino acid sequences were similar to those proteins of members of the Microviridae family were selected for further analysis. The total numbers of sequence reads showing similarity to Microviridae (abbreviated as SSM) in each library are listed in Supplementary Table S1. To compare the abundance of SSM in different species of animal or sample type, the number of SSM was normalized to reads per million unique reads (Fig. 1b-e and Supplementary Table S1). As for the fecal samples, giant panda (Ailuropoda melanoleuca) showed the lowest abundance of SSM, accounting for 0.032 per cent of the total unique sequence reads, while pig feces showed the highest percentage (i.e. 3.61%).
of SSM (Fig. 1b). The comparison of SSM percentage in fecal virome of yaks with different health status and farms suggested that the abundance of SSM was not directly related to diarrhea of yaks in this study (Fig. 1c). Among the different sample types of cattle, feces, and genital secretion contained the highest abundance (1.34%) of SSM (Fig. 1d). Among the feces, skin swab, and oral swab from each of twenty wild rats, skin swab showed SSM percent of 0.22 per cent which is higher than those in feces (0.17%) and oral swab (0.06%) (Fig. 1e).

3.2 Genomes of Microviridae in different samples

From these viromes of the samples in this study, 713 complete circular genomes and 147 contigs with complete major capsid protein (VP1) and replication protein (VP4) of Microviridae were generated by de novo assembly. The number of Microviridae genomes from pigs (Sus scrofa domestica) was 461, accounting for 53.5 per cent of the genomes, followed by cattle (n = 98), yak (Bos grunniens) (n = 53), dog (Canis lupus familiaris) (n = 42), and others (Supplementary Fig. S1a). Based on the 713 complete circular genomes of Microviridae, the genome sizes ranged from 3,143 to 8,312 bp with 19 genomes <4.1 kb and 128 genomes >6.1 kb (Fig. 1f), significantly exceeding the known Microviridae genome size range of 4.4–6.1 kb (King et al. 2012). The characteristic genes encoding the VP1 and replication protein (VP4) were identified in all the 860 complete or near complete genomes in this study. Out of the 713 complete circular genomes, 569 genomes possessed VP1, VP4, VP2 (DNA pilot protein), and VP3 (internal scaffolding protein), where 126 genomes contained the gene order of VP4–VP1–VP2–VP3 while 433 genomes had the gene order of VP4–VP3–VP1–VP2. Two of the complete genomes only contained VP1, VP2, and VP4, and the remaining 152 genomes had unclear genome structure which all contained VP1 and VP4 while lacking VP2 and VP3 instead containing unassigned ORFs at equivalent positions. Among the 569 genomes possessing VP1, VP2, VP3, and VP4, 280 genomes contained VP5 which has function of mediating synthesis of viral ssDNA (Doore and Fane 2016), where 276 genomes had VP5 located behind VP4 and 4 genomes had VP5 before VP4. Gene order of these genomes was shown in Supplementary Table S2 and Supplementary Fig. S1b.

3.3 Divergence of VP1 in genomes of Microviridae

The well-conserved major capsid protein VP1 of Microviridae can be used as a phylogenetic marker facilitating the classification (Hopkins et al. 2014; Quaiser et al. 2015). By BLASTp search in GenBank based on the VP1 amino acid sequence of the 860 Microviridae strains, the sequence identities between our sequenced Microviridae and their best matches in GenBank were delineated (Fig. 1g), which indicated that 204 strains showed >60 per cent sequence identities to their best matches in GenBank and the other 656 stains shared <60 per cent identity.
Figure 4. Phylogenetic analysis of Microviridae from virome in different species or group of animals. Animals species or group names are shown under each tree. Phylogenetic tree based on VP1 amino acid sequence of the Microviridae strains identified in this study and twenty-two representative members of the previously identified subfamilies. Within the tree, the twenty-two representative members of the previously confirmed subfamilies in Microviridae are marked with black dots and the clades in which they are located are shaded with different colors. The subfamily names are shown in text box beside their corresponding shaded colors. The scale bar indicates amino acid substitutions per site. In the phylogenetic analysis based on sequences from cattle (panel a), different sample types are marked with different color dots. In the phylogenetic analysis based on sequences from birds (panel b) or non-human primates (panel e), different species of animals are marked with different color dots.
with the best matches including 159 strains <30 per cent identity, suggesting new putative clades in the Microviridae family. Based on the names of best matched viruses, 408 of these genomes belonged to species in the subfamily Gokushovirinae, accounting for 47.4 per cent of the Microviridae strains identified here (Supplementary Fig. 51c and Supplementary Table S2). Pairwise sequence comparison of the 860 amino acid sequences of VP1 identified in this study showed that they shared sequence identities ranging from 10.3 to 100 per cent, where >70 per cent of these identities showed >50 per cent, indicating the highly genetic diversity of these Microviridae (Fig. 1h).

3.4 Phylogenetic analysis based on VP1 proteins
To further assess the genetic diversity of the 860 Microviridae strains, phylogenetic analysis was performed using the amino acid sequences of the major capsid protein VP1, where 18 representative sequences of already identified Microviridae were also included (Fig. 2a). Results indicated that 437 Microviridae genomes clustered within clades related to Gokushovirinae, 23 genomes fell into the cluster which also included Stokavirinae, Pichovirinae, Aravirinae, and Parabacteroides-phage, 9 strains were related to Bullavirinae though showing highly divergence with each other, 17 genomes clustered with the dragonfly-associated microphage which was suggested to be a potentially novel subfamily (Group D) (Quaiser et al. 2015), 27 sequences were clustered within the clade of Alpavirinae, the other 347 Microviridae genomes did not clustered within any known families but formed several potentially new clades (Fig. 2a). From each of the potential new clades, two or three representative genomes were selected for a new phylogenetic analysis which confirmed that several groups of strains place outside of existing subfamilies in Microviridae (Fig. 2b). To investigate whether the 860 Microviridae genomes clustered according to their isolation sources and geographic location, metadata of host, and geographic information were added to each strains and phylogenetic analysis was performed, which indicated that overall these Microviridae failed to cluster according to animal sources and geographic sampling sites, although a small number of strains from the sample host or sampling site did cluster phylogenetically (Fig. 2c and d).

Phylogenetic analysis based on those 410 strains from pigs located in four different sampling pig farms in Shanghai indicated that these Microviridae genomes did not cluster according to farms (Fig. 3a), where about 50 per cent of the strains showed relationship to Gokushovirinae while the remaining genomes fell into several potential new subfamilies. Out of the 102 pig feces barcodes, 88 were constructed using individual samples, where different Microviridae genomes were detected in a single barcode, suggesting the presence of multiple viral strains in a single animal. Four of the barcodes contained 19–22 different genomes that were used for phylogenetic analysis to investigate the relationship among different Microviridae strains co-existing in the same animal. Each of the four pigs showed >10 divergent strains related to Gokushovirinae, which also suggested that even in single animal multiple divergent genomes from the same subfamily of Microviridae could be present (Fig. 3b). Besides pig, seven of the other groups of animals and human contained a large number of different Microviridae genomes which were also subjected to phylogenetic analysis based on each species or group of animals, respectively (Fig. 4a–i). Although Gokushovirinae genomes were dominant among members of the Microviridae family in most of the animal species, they only accounted for a fraction (17.3%) of the 98 Microviridae genomes from cattle.

4. Discussion
The Microviridae, being secondary actors in environmental viral communities (Reyes et al. 2012; Scarpellini et al. 2015), is one of the most globally ubiquitous and highly diverse virus families (Tucker et al. 2011; Roux et al. 2012; Hopkins et al. 2014; Quaiser et al. 2015). The discovery of novel bacteriophages is classically dependent on plaque assays of bacterial lawns, such as the historically known bacteriophage T7 (Yin 1993; Abedon 2018), and some single-stranded DNA (ssDNA) phages were also identified through culture methods recently (Nasu et al. 2000; Murugaiyan et al. 2011). As a result, the majority of previously identified phages were isolated from bacteria belonging to easily culturable groups. So far, most of the recognized phages are tailed, dsDNA phages belonging to the order Caudovirales (Adams et al. 2016), while information on the ssDNA is still limited, with members of the Microviridae family only representing about 2 per cent of the phages in the ICTV database. Many studies have demonstrated the ubiquity of Microviridae genomes across habitats (marine, freshwater, wastewater, sediment) and global regions (Antarctic to subtropical), especially those related to the Gokushovirinae lineage (Tucker et al. 2011; Roux et al. 2012; Hopkins et al. 2014; Quaiser et al. 2015). With increasing number of novel Microviridae genomes, more potential subfamilies have been suggested including Alpavirinae, which seem to be mainly associated with human microbiota (Krupovic and Forterre 2011), Pichovirinae, which have a broader environmental range (Roux et al. 2012), and Stokavirinae, Aravirinae, and Group D, which were identified from sphagnum-dominated peatlands (Quaiser et al. 2015). In the present study, using viral metagenomics, 860 complete or near complete genomes showing sequence similarity to Microviridae in the virome of different types of samples from human, mammals, and birds, and digestive tract of oyster and fresh water were acquired. These genomes showed considerable variations, even in the sample from a single animal showing multiple co-existence of different genomes. Sequence comparison and phylogenetic analysis based on the major capsid protein, provided insights into the evolution and genetic diversity of the Microviridae family, suggesting that although the Gokushovirinae subfamily was dominant some new subfamilies might exist. Identification of the corresponding bacterial hosts should help elucidate the ecological and functional significance of these Microviridae subfamilies.

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Data availability
The raw sequence reads from the metagenomic libraries were deposited in the Short Read Archive of GenBank database and the accession numbers were listed in Supplementary Table S1. The 860 Microviridae genomes identified in this study were submitted to GenBank and the accession numbers were listed in Supplementary Table S2.

Supplementary data
Supplementary data are available at Virus Evolution online.
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