Metagenomic Analysis of the Virome in Lung Tissues and Guts of Wild Mice

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Research

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Abstract

Background

Mice, as host animals of a variety of pathogens, can spread 60 kinds of human diseases including more than ten families of viruses including Poxviridae, Herpesviridae, and so on.

Methods

In this study, lung tissues and gut samples of 7-week-old mice from outdoor environments were sequenced using metagenomics, and an abundance of virome information was acquired.

Results

A total of 82 families of mammalian viruses, plant viruses, insect viruses, and phages were detected. Among the top 10 most abundant families were the RNA viruses Orthomyxoviridae, Picornaviridae, Bunyaviridae, and Arenaviridae, the DNA virus Herpesviridae, the insect viruses Nodaviridae and Baculoviridae, the plant virus Tombusviridae, and the phage Myoviridae. Except for Myoviridae, whose abundance in guts was higher than in lung tissues, the abundance of viruses in the lung tissues and guts showed no significant difference.

Conclusions

The data obtained in this study provided an overview of the viral community present in these mice samples, revealing some mouse-associated viruses closely related to known human or animal pathogens. Strengthening our understanding of unclassified viruses in mice in the natural environment could provide scientific guidance for the prevention and control of new viral outbreaks that can spread via rodents.

1. Introduction

Mice, as host animals of a variety of pathogens, can spread 60 kinds of human diseases including more than ten families of viruses including Poxviridae, Herpesviridae, and so on. For instance rodents are the natural host of Hantavirus, which commonly causes HFRS in Asia and Europe and HPS in North and South America [1]. Rodents are also the natural host of Arenavirus, which causes Lassa Fever, a condition with high mortality in humans [2]. Arenavirus belongs to the family of Arenaviruses, the representative virus of which is the Lymphocytic choriomeningitis virus, which is distributed globally[3]. The main reason for this worldwide distribution is that Mus musculus are the primary host animals for this virus. Therefore, mice carry many disease-causing viruses and are the cause for increasing concern. While mice carry these viruses, they do not show any clinical symptoms, and thus, it is easy to ignore the potential threat of natural viruses to human and animals. Therefore, strengthening the research of wildlife etiology to understand the existence of viruses and epidemic conditions in nature is important for the prevention and control of new viral epidemics and outbreaks.

Traditional virology research methods are limited to tissue pathology and virus culture, which makes it difficult to study viruses that cannot be cultured. Metagenomics sequencing technologies make it possible to find new viruses from the angle of the genome. In a few short years, metagenomics research has penetrated into all areas of potential viral life, including ocean, soil, hot springs, human oral cavities and the gastrointestinal tract[4–9]. Surprisingly, in nearshore marine environments, 65% of the detected virus sequences were previously unknown and genotype data revealed a total of 5000 viral species[7]. Horse fecal samples were sequenced using high-throughput sequencing technologies and 68% of virus sequences identified were previously unknown while the genotype data identified up to 1000 different viral species[8]. Finally, feces samples from humans contained as many as 1200 unique viral genotypes identified through metagenomics sequencing, and rare and new intestinal viruses were found [10–12].

Zhang et al., BLASTed 36,769 RNA virus sequences of samples from the healthy human intestinal tract and found that most sequences were similar to plant RNA viruses[13]. Day et al., used metagenomics to analyze the virome of turkeys suffering from enterovirus syndrome and found many new unidentified viral species[14]. Bats are the natural host of many zoonotic viruses; Li and Donaldson[15, 16] collected samples from the intestine of North American bat species and analyzed them using metagenomics. They found that the intestines of the bats contained a rich pool of viruses with not only viruses that can infect animals, but also many new plant and insect viruses. In addition, the study by Donaldson identified three new strains of genetic type I coronaviruses. He also used virus metagenomics technology to analyze the virus community of fecal samples of bats from different areas in China and showed that the bacterium and virus community accounted for 60% of species in the feces, where insect viruses accounted for 35%, while vertebrate, plant and protozoan viruses accounted for about 5% of all viruses.

In this study, gut and lung tissue were collected from 3 Clethrionomys rufocanus organisms, which are wild representatives of mice, to assess the variety of viruses carried by the mice. Metagenomic analysis was then conducted to screen the viromes of these samples. Herein, we outline the viral spectrum within these mouse samples. These data offer new clues for tracing the sources of important viral pathogens that can cause human and animal disease.

2. Materials And Methods

2.1. Sample processing

Lung tissues and gut samples from 3 Myodes rufocanus organisms that live outdoors were homogenized, diluted in a ratio of 1:10 in PBS, made into a suspension, and vortexed for thorough mixing. The samples were then centrifuged at 2000 rpm for 10 min at 4 °C, and supernatants were transferred to a fresh tube and centrifuged for 10 min to fully remove cell debris, bacterial cells, and other impurities. The supernatants were filtered through a 0.45-μm syringe filter (Jet,Guangzhou, China) and concentrated. Filtrate was centrifuged in a SW55Ti rotor in a Beckman ultracentrifuge at 45,000 r/min for 2 h. The precipitates were resuspended in PBS and filtered with 0.45-μm syringe filter; then, samples were stored at -80 ℃ until subsequent analyses.

2.2. Nuclease treatment and RNA /DNA extraction

To reduce the free nucleic acid content, which can interfere with the experiment, 116 μL of filtration liquid was added to each sample along with 14 U of DNase (TAKARA, DaLian, China), 25 U of nuclease (Novagen, Darmstadt, Germany), 20 U of RNase I (Promega, Madison, WI) and 10 × Turbo DNase buffer (TAKARA, DaLian, China). Samples were digested in a 37 ℃ water bath for 2 h, then processed with an Easy Pure Viral DNA/RNA Kit (TransGen Biotech, Beijing, China) using the manufacturer's protocol to extract viral DNA and RNA. Viral DNA and RNA were eluted to a final volume of 60 μL and stored at -80 °C until further use.

2.3. Reverse transcription and PCR

First, 6 μL of the viral nucleic acid samples was added to a tube with 1 μL of 25 μmol/L 6mer random primers with a 20-bp anchor sequence. The mixtures were incubated at 70℃ for 10 min, then immediately put in an ice bath for 2 min. Then, 0.5 μL of dNTP mixture (10 mmol), 0.25 μL RNase Inhibitor (400 U/μL), 0.25 μL of RTase M-MLV (200 U/ul), 2 μL of 5 × buffer, and 1 μL of ddH_2O were added, and the mixtures were incubated at 42℃ for 60 min, 30 ℃ for 10 min, and 75 ℃ for 15 min for cDNA synthesis, and 85 ℃ for 10 min to inactivate reverse transcriptase. Next, 5 U of DNA polymerase Klenow fragment was added and the mixtures were incubated at 37 ℃ for 60 min to prepare synthetic double-stranded cDNA, and then at 75 °C for 10 min. Then, 2 U of shrimp alkaline phosphatase and 2.5 U of Exonuclease I were added into the system, followed by incubation at 37℃ for 60 min to remove redundant primers and free nucleotides. The mixture was then incubated at 72 °C for 15 min. Next, 10 μL of the template, 2 μL of 10 μmol/L anchor sequence primers, 5 μL of 10 × AccuPrime buffer, and 1 μL of Accuprime Taq DNA Polymerase was added, and ddH_2O was used to attain a total volume of 50 μL to carry out sequence-independent single primer amplification (SISPA). The thermal cycler profile was as follows: 72 °C for 10 min, 95 °C for 10 min, 40 cycles of 95 °C for 30 s, 51.9 °C for 40 s, and 72 °C for 90 s. Amplified products were purified using an AxyPrep DNA Gel Extraction Kit (AxyGen, California, USA) and solubilized in 40 μL of TE buffer.

2.4. Sample testing and Library construction

DNA concentration was measured using a Qubit® dsDNA Assay Kit in the Qubit® 2.0 Flurometer (Life Technologies, CA, USA). Samples with OD values between 1.8 ~ 2.0 and DNA contents above 1 μg were used to construct a library. A total amount of 1 μg of DNA per sample was used as input material for the DNA sample preparations. Sequencing libraries were generated using the NEBNext® Ultra™DNA Library Prep Kit for Illumina (NEB, USA) following manufacturer's recommendations and index codes were added to attribute sequences to each sample. Briefly, the DNA samples were fragmented by sonication to a size of 300 bp, and then, the DNA fragments were end-polished, A-tailed, and ligated with the full-length adaptor for Illumina sequencing for further PCR amplification. Finally, PCR products were purified (AMPure XP system) and libraries were analyzed for size distribution using an Agilent2100 Bioanalyzer and quantified using real-time PCR.

2.5. Sequencing

The clustering of the index-coded samples was performed on a cBot Cluster Generation System according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina HiSeq2500 platform and paired-end reads were generated.

2.6. Species annotation and the abundance Analyzing

Pretreated Clean Data of all the samples were compared to the reference genome of viruses in the NCBI database (Refseq), virus data in the NT (Version: 2014-10-19) database and the ACLAME database for BLAST (setting threshold to value ≤ 1e-3). The results from each database were merged for the comparison results of a read, and the values with the highest scores were chosen for the comparison results of reads. Using MAGA software to analyze phylogenetic relationships, an evolutionary tree was made based on the neighbor-joining method of maximum composite likelihood with the bootstrap set to 1,000 repeats.

Based on the numbers of reads and the abundance of information in each sample for each classification level (phylum, class, order, family, and genus), statistical analysis and visual display was performed.

2.7. Statistical analysis

Metastat was used to analyze the top 10 abundant taxonomic sequence tags of the three samples. When the p-value was less than 0.05, the differences were considered to be statistically significant.

3. Results

3.1. Sequencing and quality control

Sequencing of DNA and RNA extracted from lung tissues and gut samples of 3 wild Myodes rufocanus organisms was performed. The length of the insert size was 350 bp. Bases showing overlapping information and low mass, and bases that were not measured were excluded and the total numbers of clean data obtained from the six samples (three lung and three gut samples) were 238493, 209033, 199432, 239177, 200730, and 214870, respectively. Sequencing data quality was distributed in the quality score Q20 so as to ensure a normal order of the subsequent advanced analysis. The clean sequence tags were subjected to redundancy processing using the mothur software to obtain unique sequence tags. The percentages of effective sequences of the six samples were 95.242%, 93.561%, 97.509%, 97.258%, 93.121%, and 94.622%, respectively (Table 1).

3.2. Distribution of the samples based on family-level classification

Pretreated Clean Data of all the samples was compared with the reference genome of viruses in the NCBI database, NT database, and ACLAME database to obtain an annotation to each level (from Kingdom to Species).

In total, 82 families of mammalian viruses, plant viruses, phages, insect viruses and fungal viruses were found. An overview of the reads of the top 35 families of viruses in each sample is shown in Fig. 1. In addition, an overview of the classification of the identified mouse viruses in each sample from Kingdom to Species is shown in Fig. 2 (a-f).

The top 10 most widely distributed families of viruses were Orthomyxoviridae, Picobirnaviridae, Herpesviridae, Nodaviridae, Bunyaviridae, Arenaviridae, Myoviridae, Tombusviridae, Unclassified, and Baculoviridae. The reads related to the family of Orthomyxoviridae comprised the largest proportion of viruses, especially in lung tissue samples, most of which were classified into the genus Influenzavirus A. The diverse reads related to these families occupied 25–45% of the total viral sequence reads. It is worth mentioning that lung1 and lung2 did not contain any members from Tombusviridae (Fig. 3a,b).

3.3. Single-stranded RNA viruses identified in mouse samples (Orthomyxoviridae, Picornaviridae, Bunyaviridae, and Arenaviridae)

The family Orthomyxoviridae is a group of large enveloped and segmented viruses with negative-sense single stranded RNA genomes (~ 13.6 kb in length). The members of this family can cause animal acute upper respiratory tract infection, and can spread quickly by air. Thus, there are cyclical pandemics throughout the world of species from the Orthomyxoviridae family [17]. The host of influenza viruses and their route of transmission is also important in human health, though mice can also be infected with influenza virus. In this study, the reads related to the family Orthomyxoviridae comprised the largest proportion of viruses with their percentages in each sample being: lung1: 45.04%, lung2: 51.57%, lung3: 41.08%, gut1: 41.9%, gut2: 27.59%, and gut3: 22.1%. The family were assigned to the genus of Influenzavirus A, with species of influenza A virus.

Picornaviridae are small, non-enveloped, positive single-stranded RNA viruses with a genome of 7–9 kb in size. The members of the family of Picornaviridae cause a wide variety of vertebrate hosts mucocutaneous, encephalic, cardiac, hepatic, neurological and respiratory diseases [18]. The Picornaviridae family viruses existed in all six samples and were assigned to the genus of Picobirnavirus, with species of human picobirnavirus, microtus picobirnavirus V-111_USA_2008, and fox picobirnavirus.

The family of Bunyavirus is a group of spherical enveloped viruses with negative-sense single-stranded RNA genomes (250–450 kd in length). These viruses have strong infectivity, wide distribution, and high fatality rate, and can cause serious infectious diseases in humans and animals[19]. As one of the biggest RNA virus families, it includes five genera: Orthobunyavirus, Hantavirus, Nairovirus, Phlebovirus, and Tospovirus. Most of the members of this family such as Rift valley fever virus, Crimean-Congo hemorrhagic fever virus, La Crosse encephalitis virus, and Hantavirus, cause deadly diseases in humans. The natural host of Hantavirus is rodents, and it can cause kidney hemorrhagic fever. The virus was present in all samples of the three Myodes rufocanus organisms. Among these, the abundance in the

gut3 (3.49%) sample was higher than in the others. In samples, the viruses of this family were assigned to the genus of Orthobunyavirus, and the species Shamonda virus.

Arenavirus is an enveloped RNA virus found worldwide. The virus genome is divided into two segments. Five human pathogenic viruses of this family have been found, including Lassa fever virus (LASV), lymphocytic choriomeningitis virus, Tacaribe virus, Junin virus, and Machupo virus. Among these, Lassa fever virus,Junin virus and Machupo virus can cause severe disease with a high mortality rate. Lassa fever virus is highly contagious and its infection is easily passed on from person to person[20]. The family of Arenavirus are commonly associated with rodents, so the prevalence of the infectious disease is closely related with the local dynamic distribution of rodents. In this study, the virus was detected in the lung tissue and feces of the 3 mice. The viruses in this family were assigned to the genus Mammarenavirus, and species Lassa mammarenavirus.

3.4. Bat main DNA viruses (Herpesviridae)

Herpesviridae family are enveloped, double-strand DNA viruses which are divided into three genera based on phylogenetic clustering: alphaherpesvirus, betaherpesvirus, and gammaherpesvirus. α-herpes viruses proliferate quickly and can cause pathological changes within cells; they include herpes simplex virus and varicella zoster virus. The growth cycle of β-herpes virus is longer and these viruses infect cells to form giant cells, such as cytomegalovirus. Target cells of γ-herpes virus infection are lymphoid cells, and infection can lead to lymphoid hyperplasia, such as the case with Epstein-Barr virus. This family can be detected in the lung tissues and gut samples of the 3 mice. The viruses in this family were assigned to the genera: Cytomegalovirus, Varicellovirus, and Mardivirus, and the following species: Cercopithecine herpesvirus 5 and Gallid herpesvirus 2.

3.5. Other rare mouse viruses (Nodaviridae, Baculoviridae, Tombusviridae, Myoviridae, and Unclassified)

Insect virus (Nodaviridae, Baculoviridae), plant virus (Tombusviridae), phages (Myoviridae) and unclassified viruses were identified in the samples. The viruses in the family of Nodaviridae were assigned to the genus Alphanodavirus and Betanodavirus, and species of Pariacoto virus and Barfin flounder nervous necrosis virus. The viruses in the family of Tombusviridae were assigned to the genus Tombusvirus.

4. Discussion

Metagenomics has provided system development descriptions and functional analysis for the virome of many species. Compared with other molecular biology techniques, the data collected through high-throughput sequencing technologies is more direct and more comprehensive[21].

Zhang Yongzhen et al. detected a Puumala-like virus in Myodes rufocanus; this virus showed a 91.7–97% amino acid sequence homology with the S segment of Puumala virus from the JiLin province of China[22]. Zhang Yunzhi et al.used random PCR and 454 high-throughput sequencing methods to study

628 feces samples from different areas of the YunNan province. Their preliminary results show that there are a lot of viruses in the mouse intestine including Coronavirus, Paramyxovirus, Adenovirus, and Rubivirus. There are also many new mouse sources of DNA and RNA viruses[23]. Phan et al. used metagenomics recently to study intestinal viruses in 105 mice in the United States and retrieved 26,846 virus sequences (> 100 bp) including more than 20 unique kinds of viruses such as Circoviridae, Geminiviridae, and Nanoviridae[23]. New mouse Papilloma virus, wreath virus, kobuvirus, small RNA viruses, micro-RNA viruses, stellate virus, adenovirus and adeno-associated virus also were found. Thus, at least in the United States, rodents carry many unknown new viruses which traditional tissue culture methods are unable to find, thus opening a new field of vision for disease prevention and control.

In this study, we conducted a viral metagenomic analysis of fecal and lung tissue samples from mice using the Solexa sequencing technique (Illumina). The data analysis indicated that the most abundant sequences were related to mammalian viruses, insect viruses, plant viruses, and phages.

This report suggests that mice harbor a large spectrum of mammalian viruses, especially Influenza A virus, from the family Orthomyxoviridae in both feces and lung tissues. Additionally, there is no significant difference between the two tissues in terms of viral species, implying that if humans have close contact with rodents, they may be infected with influenza virus or other viruses. The natural reservoir of Hantavirus and Arenavirus are mice, and they can cause serious infectious diseases in humans and animals.

In lung tissues and gut samples from wild-life mice, insect viruses (Nodaviridae, Baculoviridae) and plant viruses (Tombusviridae) were found. The presence of these viruses may be related to the survival environment of mice and the intake of food. In addition, phages (Myoviridae) were also was detected and the abundance of Myoviridae in the lungs was significantly higher than in the feces ($p = 0.037$). It is worth mentioning that the virus does not exist in lung2 and lung1 samples and this may be due to the presence of different viral species in the lungs and lungs. The presence of a group of unclassified viruses implies that many of the viruses in rodents are unknown and require further exploration.

5. Conclusion

The metagenomics approach can greatly improve our understanding of the diversity of viruses in mice. Using metagenomics technology, this study analyzed the composition and abundance of virus genome in lung tissues and guts from 3 wild-life mice. This strategy could be extended to other wildlife or livestock samples worldwide, ultimately increasing our knowledge of the viral population and ecological community, and thus minimizing the impact of potential wildlife-associated viruses on public health by providing meaningful basic data.

Abbreviations

HFRS:Hemorrhagic fever of renal syndrome; HPS:pulmonary syndrome ;LASV:Lassa fever virus

Declarations

Availability of data and materials

The datasets analyzed during the current study available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The study was approved by Harbin Veterinary Research Institute and performed in accordance with animal ethics guidelines and approved protocols. The animal Ethics Committee approval number is Heilongjiang SYXK-2006-032.

Consent for publication

Not applicable

Author Contributions: H.-C.Y., H.-Y.C., wrote the paper. L.-L.Z., and T.-F.L. prepared the graphs and table. All authors read and approved the fifinal manuscript.

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Conflicts of Interest: The authors declare that there is no conflflict of interest regarding the publication of this article

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Table 1

Due to technical limitations, table 1 is only available as a download in the supplemental files section.

Table 1. Output of data statistics

Figures

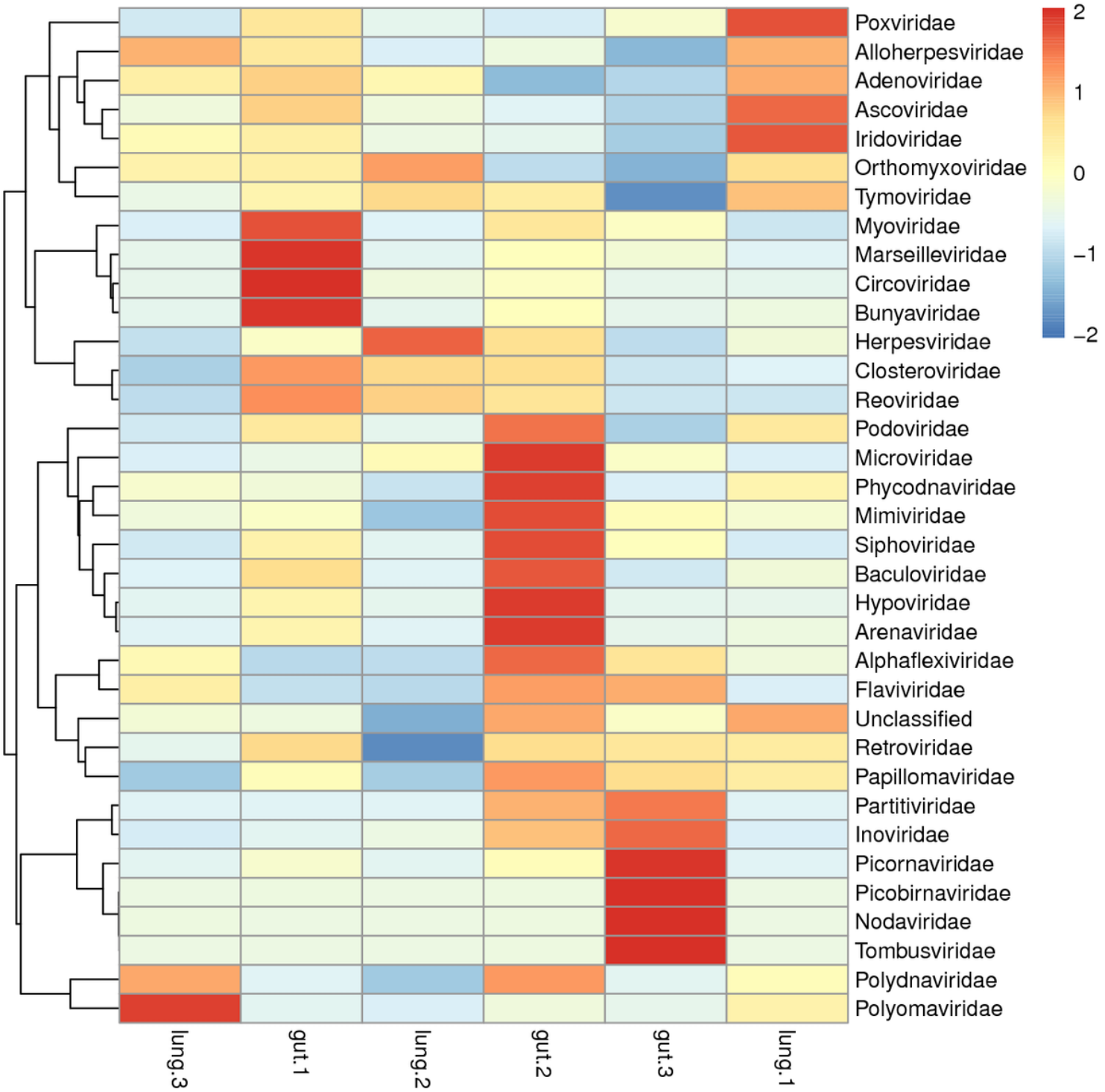

Figure 1

Heatmap based on the normalized sequence reads of 35 families of viruses in each pooled sample. The horizontal axis lists the sample names. The Y-axis shows species information. The diagram on the left side of the clustering tree is a species tree. The color of the boxes represent the metagenomic sequencing reads observed.

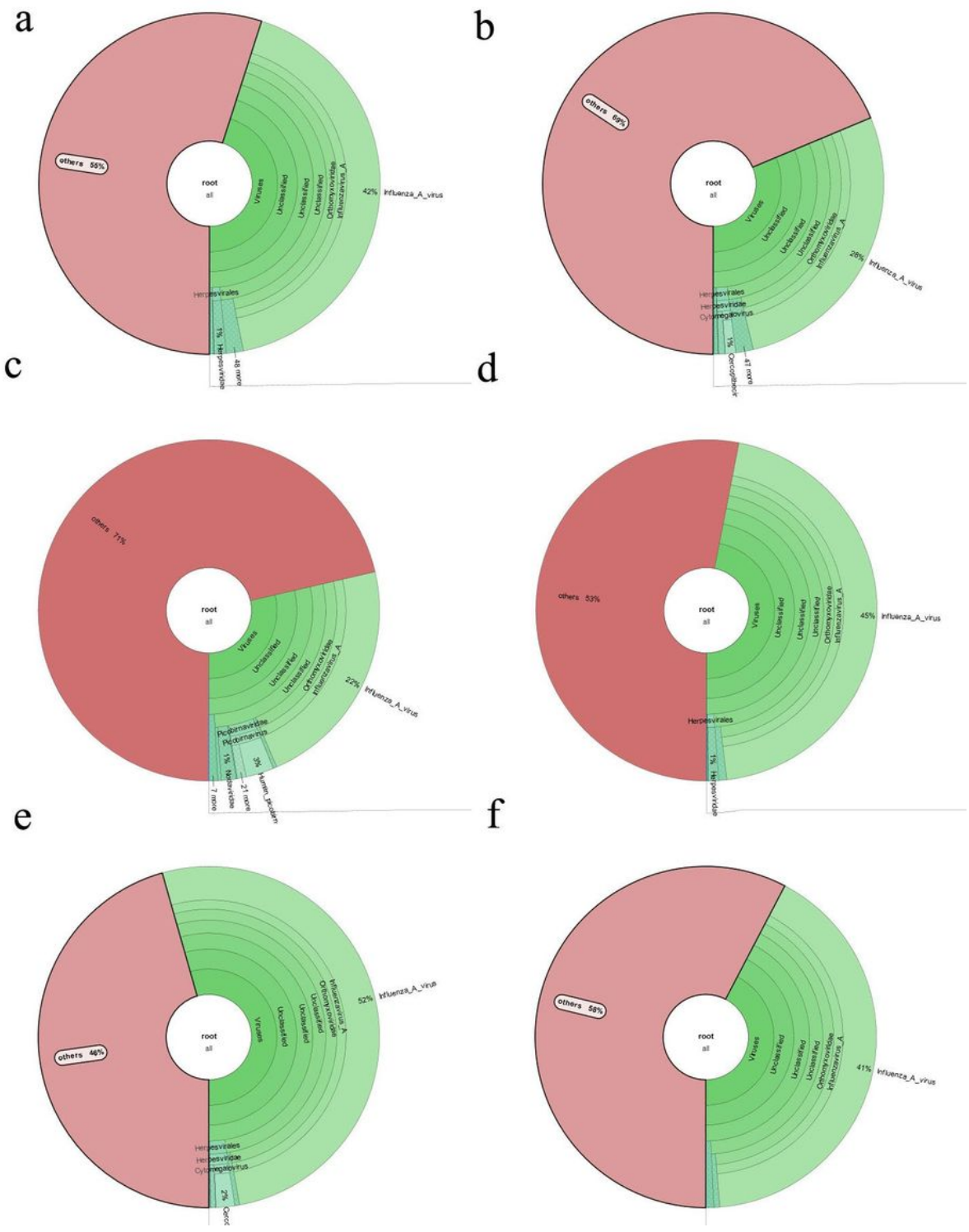

Figure 2

(a-f) Overview of the classification of the identified mice viruses from each sample in this study from kingdom to species. “Others” indicates the sum of the relative abundances of all the other levels (from kingdom to species) and are labeled in a pink box.”a-f “refer to gut1,gut2,gut3,lung1,lung2 and lung3 respectively.

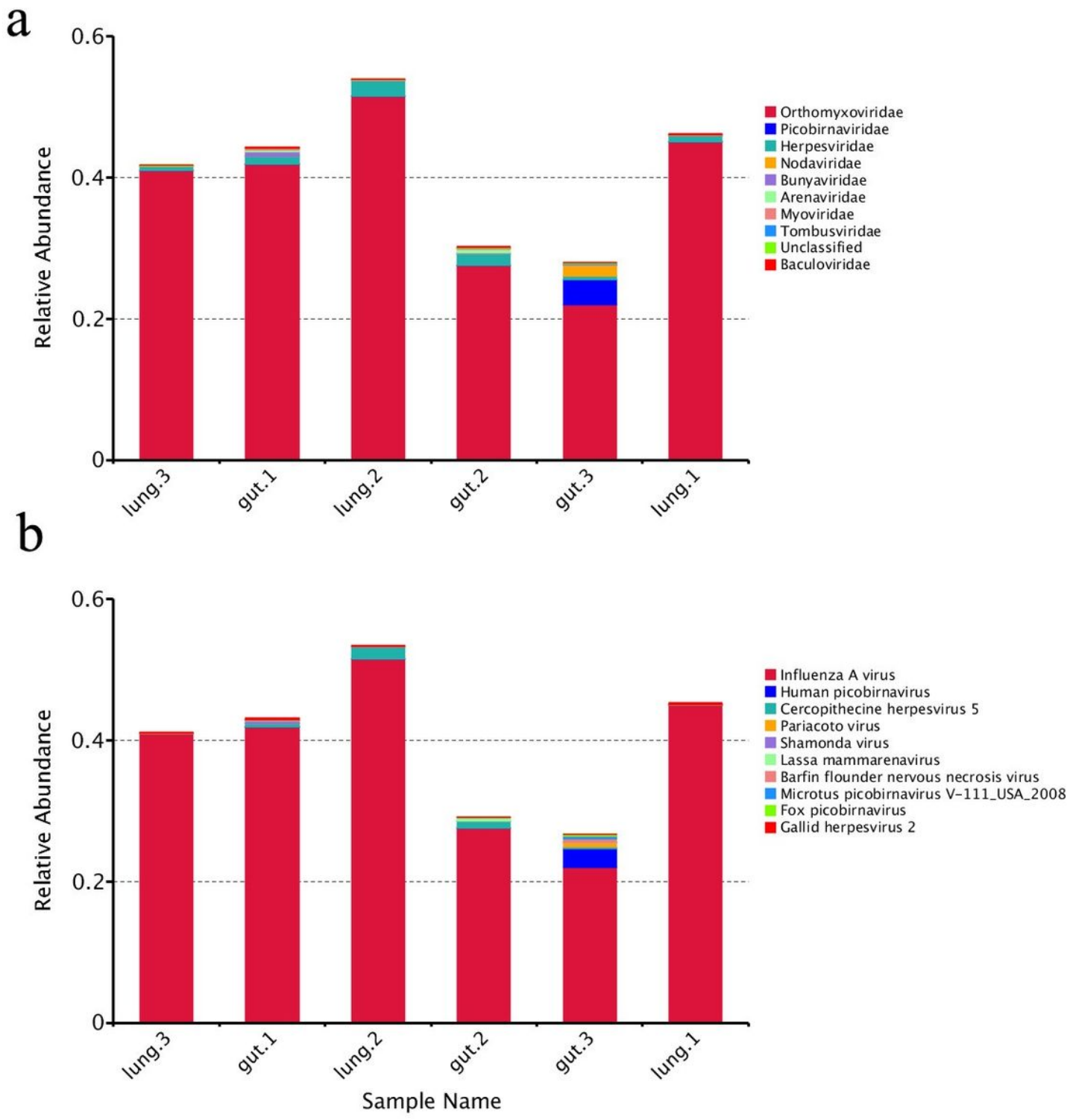

Figure 3

a and b represent the relative abundance of families and species, respectively, identified in each sample. The Y axis shows the ratio annotation to a certain type of species, while the X axis shows the sample name. The corresponding color blocks show the species category (legend on the right side).

Supplementary Files

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- Table1.png