Original Article

Metagenomic Analysis of Environmental Samples from Wildlife Rescue Station at Poyang Lake, China*

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Abstract

Objective To improve the understanding of the virome and bacterial microbiome in the wildlife rescue station of Poyang Lake, China.

Methods Ten smear samples were collected in March 2019. Metagenomic sequencing was performed to delineate bacterial and viral diversity. Taxonomic analysis was performed using the Kraken2 and Bracken methods. A maximum-likelihood tree was constructed based on the RNA-dependent RNA polymerase (RdRp) region of picornavirus.

Results We identified 363 bacterial and 6 viral families. A significant difference in microbial and viral abundance was found between samples S01–S09 and S10. In S01–S09, members of *Flavobacteriia* and *Gammaproteobacteria* were the most prevalent, while in S10, the most prevalent bacteria class was *Actinomycetia*. Among S01–S09, members of *Myoviridae* and *Herelleviridae* were the most prevalent, while the dominant virus family of S10 was *Picornaviridae*. The full genome of the pigeon mesivirus-like virus (NC-BM-233) was recovered from S10 and contained an open reading frame of 8,124 nt. It showed the best hit to the pigeon mesivirus 2 polyprotein, with 84.10% amino acid identity. Phylogenetic analysis showed that RdRp clustered into *Megrivirus B*.

Conclusion This study provides an initial assessment of the bacteria and viruses in the cage-smeared samples, broadens our knowledge of viral and bacterial diversity, and is a way to discover potential pathogens in wild birds

Key words: Metagenomics; Virome; *Megrivirus B*; *Picornaviridae*; Poyang Lake; Wildlife Rescue Station; Wild Bird

Biomed Environ Sci, 2023; 36(7): 595-603 *doi: 10.3967/bes2023.087* *ISSN: 0895-3988*

www.besjournal.com (full text) *CN: 11-2816/Q*

INTRODUCTION

Poyang Lake is one of the most important wintering sites for waterfowl along the migration route from East Asia to Australia. This area is a Ramsar site, a wetland site designated internationally important under the Ramsar Convention[1]. Hundreds of thousands of migratory birds, including rare and endangered species, from Siberia, Mongolia, Japan, and northeastern and northwestern China overwinter at Poyang Lake annually[2]. Bird monitoring showed that over 98% of the global population of Siberian cranes, 50% of swan geese, and 50% of white-napped cranes overwinter at Poyang Lake. In addition, 80% of the global population of oriental white storks is also found here in winter[3,4].

Wild waterfowl hosts a vast diversity of well-

*This research was funded by the National Natural Science Foundation of China [81961128002].

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known and many potential pathogens[5,6]. Farmers raise poultry in the water system of the Poyang Lake area[7]. Thus, migratory birds may share food, water, and even habitats with domestic poultry, creating opportunities for pathogen transmission[7]. Specifically, the effective habitat area for migratory birds to forage in dry seasons (autumn and winter) is greatly reduced[8,9], resulting in significantly more waterfowl foraging on farmland around the lake than in wet seasons, exacerbating the spread of pathogens between domestic and wild birds[7].

The emergence of new infections poses a threat to both animal and human health. To gain a better understanding of newly emerging pathogens at Poyang Lake, we characterized the bacterial microbiome and virome diversity of samples through metagenomic analysis in the Duchang Tangkou Wildlife Rescue Station and identified a complete genome of new pigeon mesivirus-like virus.

MATERIALS AND METHODS

Sample Collection

A total of ten specimens, one surface-smearing swab per cage, were collected at the Duchang Tangkou Wildlife Rescue Station (29.209982 N, 116.463861 E) on March 10, 2019, named S01–S10. The caged bird species of S01–S08 are unknown. The species of S09 and S10 were a skylark and a red turtle dove, respectively. The swabs were immediately placed in sterile tubes at 4 °C until transported to the laboratory for storage at −75 °C.

Nucleic Acid Extraction

Total RNA was extracted from each sample using the MagMAX CORE Nucleic Acid Purification Kit (Applied Biosystems, Shanghai, China, Cat# A32702) according to the manufacturer's instructions. RNA was eluted in a final volume of 85 µL.

Library Preparation

An RNA reverse transcription kit (MatriDx Biotech Corp., Hangzhou, China, Cat# MD017) was used for library preparation. For each sample, 14 µL of RNA was mixed with 2 µL of the enzyme mixture in buffer in a 0.2 mL tube of as the first step. The total reaction volume was 20 µL. PCR using Bio-Rad T-100 cycler (Hercules, CA, USA) was conducted. The reaction conditions were as follows: 25 °C for 10 min, 50 °C for 30 min, 75 °C for 10 min, and held at 4 °C. The chain synthesis products (20 µL) were added to 2 µL of the enzyme mixture in the buffer and 18 µL nuclease-free ddH_2O in the second step. A total volume of 40 µL was used in the PCR reaction. The reaction conditions were as follows: 16 °C for 15 min and stored at 4 °C. DNA purification was performed using a DNA purification kit (magnetic bead method) (Matridx Biotech Corp., Hangzhou, China, Cat# MD012T). The 35 µL purification solution was prepared for the follow-up experiments. The cDNA concentrations were determined using a Qubit X-Green II dsDNA Quantitation Kit (Yuheng Biotech Corp., Suzhou, China, Cat# Q2038). All cDNA was diluted 1:200 with dsDNA HS Buffer.

Metagenome Sequencing

Libraries were constructed according to the manufacturer's protocol using the Metagenomic DNA Library Preparation Kit (MatriDx Biotech Corp., Hangzhou, China, Cat# MD001T). The DNA was fragmented using an enzyme. The reaction (50 µL) was incubated at 37 °C for 10 min, 75 °C for 10 min, and held at 4 °C. The adaptors were added to the fragmented DNA solution. The reaction conditions were set at 20 °C for 15 min and 75 °C for 5 min. The solutions containing adaptors were purified again. DNA was quantified using a Magic dsDNA HS Assay Kit (magic-bio, Cat #VG00537). The thermocycling conditions were 98 °C for 45 s, followed by 13 cycles of denaturation (98 °C for 15 s), annealing (60 °C for 30 s), and extension (72 °C for 30 s). Equal concentrations of the samples were added to the final pool. Libraries were pooled and sequenced (75 bp single end) using a NextSeq500 sequencer. The clean raw reads were retained by removing low-quality and low-complexity reads. All sequencing was performed by MatriDx Biotech (Hangzhou, China).

Bioinformatic Analysis and Phylogenetic Analysis

For each library, sequencing reads were trimmed using Trimomatic V0.33[10]. The obtained reads were used to perform taxonomic analysis using the Kraken2 program with default parameter settings using the Standard PlusPF database (https://benlangmead.github.io/aws-indexes/k2, 5/17/2021)[11]. Species abundance after classification with Kraken2 was re-estimated using Bracken[12]. No filtering of the host/bacterial reads was performed before taxonomic analysis. For sample comparison, principal coordinate analysis (PCoA) based on Jaccard distance was performed on the bacterial microbiome or virome counts of the Bracken outputs.

The trimmed reads of each sample were individually assembled using MEGAHIT v1.2.9 (default parameter)[13]. Prodigal V2.6.3 was used to perform gene prediction on the *de novo* results and extract the complete genes ("partial=00")[14]. The predicted complete genes were searched against the nucleotide sequence database from the National Center for Biotechnology Information (NCBI) with e-values of $1e^{-5}$ using BLAST [https://blast.ncbi.nlm.nih.gov/Blast.cgi]. The hypothetical cleavage map of the picornavirus polyprotein was derived from alignment with the closest picornaviruses.

The RNA-dependent RNA polymerase (RdRp) region of the viral gene and closely related amino acid sequences were aligned using MAFFT v7.222[15]. A phylogenetic tree was constructed using MEGA 7 based on the maximum-likelihood method, and the bootstrap value was tested with 1,000 replications[16]. The tree was edited and visualized using Interactive Tree of Life (iToL)[17].

Nucleotide Sequence Accession Numbers

Clean data and bio-samples can be found in NCBI BioProject PRJNA862611. The complete genome sequence of NC-BM-233 was submitted to GenBank and assigned the accession number OP169446.

RESULTS

Metagenomic Sequencing Overview

Ten libraries were constructed, and 120,085,728 clean sequence reads were generated. Using the Standard PlusPF database, 7,507 reads (0.06‰ of the total reads) were assigned to *Viruses* by Kraken2 and Bracken, and 45,871,237 reads (38% of the total reads) were assigned to *Bacteria* (Table 1).

Rarefaction Analysis

We examined the species richness of the identified taxa in ten samples. The number of observed species in S10 was the highest, followed by that in S06 and S04. In contrast, we identified a relatively low species abundance in S08 (Figure 1).

Bacterial Microbiome Diversity

Taxonomic analysis of the bacterial microbiome in ten samples was performed using Kraken2 and Bracken, and the visualization of classification results is shown as Sankey diagrams (Supplementary Figure S1, available in www.besjournal.com). A total of 1,223 bacterial genera from 34 phyla, 71 classes, 162 orders, and 363 families were identified. The *Bacteroidetes* phylum comprised most of the bacterial microbiome, with an abundance of 58.52%, followed by *Proteobacteria* (34.53%), *Firmicutes* (3.28%), *Actinobacteria* (1.94%), and *Fusobacteria* (1.59%). The class *Flavobacteriia* was the most abundant (52.59%), followed by *Gammaproteobacteria* (32.40%), *Sphingobacteriia* (5.28%), *Bacilli* (2.66%), *Actinomycetia* (1.92%), *Betaproteobacteria* (1.71%), *Fusobacteriia* (1.59%), *Clostridia* (0.50%), *Bacteroidia* (0.29%), and *Alphaproteobacteria* (0.22%) (Figure 2A, Supplementary Table S1, available in www.besjournal.com).

We examined the bacterial composition of each sample at the class, order, and family levels. Among S01 to S09, *Flavobacteriia* and *Gammaproteobacteria* classes were the most abundant, while *Actinomycetia* accounted for the largest proportion in S10 (Figure 2B). Among S01–S09, orders *Flavobacteriales*,

Table 1. Overview of reads of samples

Sample	Reads count	Viruses reads (‰)	Bacteria reads (%)	Unclassified reads (%)
S01	14,189,681	638 (0.04)	5,592,952 (39.42)	8,594,075 (60.57)
S02	12,396,722	139 (0.01)	5,895,415 (47.56)	6,499,171 (52.43)
S03	12,665,698	891 (0.07)	5,171,548 (40.83)	7,487,318 (59.11)
S04	12,730,271	696 (0.05)	4,067,597 (31.95)	8,634,041 (67.82)
S05	12,628,298	2,237 (0.18)	5,446,785 (43.13)	7,162,035 (56.71)
S06	12,671,688	612 (0.05)	4,943,276 (39.01)	7,692,390 (60.71)
S07	12,451,945	168 (0.01)	5,156,039 (41.41)	7,288,344 (58.53)
S08	11,379,174	754 (0.07)	4,527,355 (39.79)	6,836,391 (60.08)
S09	10,539,865	420 (0.04)	4,189,283 (39.75)	6,348,867 (60.24)
S10	8,432,386	952 (0.11)	880,987 (10.45)	7,473,206 (88.63)

Enterobacterales, and *Pseudomonadales* were the most abundant, and at the family level, *Flavobacteriaceae* and *Enterobacteriaceae* were the most abundant (Figure 2A, Supplementary Figure S1). In S10, *Actinomycetales* and *Micrococcales* accounted for the largest proportion, and at the family level, *Actinomycetaceae* and *Microbacteriaceae* accounted for the largest proportion (Figure 2C–D, Supplementary Figure S1).

We used the Shannon and Simpson indices to illustrate bacterial microbiome diversity based on the Bracken output (Supplementary Figure S2A–B, available in www.besjournal.com). S10 was the only outlier of the bacterial microbiome Shannon index and had the highest Simpson index value among all samples. To further assess the differences between samples, PCoA results revealed that S10 differed from the other samples in the bacterial microbiome component (Supplementary Figure S2C).

Viral Diversity

Taxonomic analysis of the virus results is shown in Sankey diagrams (Supplementary Figure S3, available in www.besjournal.com). A total of 38 virus species from 2 orders, 6 families, and 13 genera were identified. These two orders, *Picornavirales*

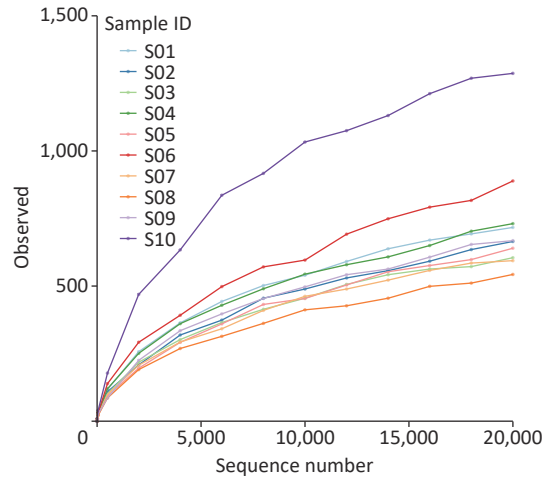

Figure 1. Rarefaction curve analysis of observed species on each sample.

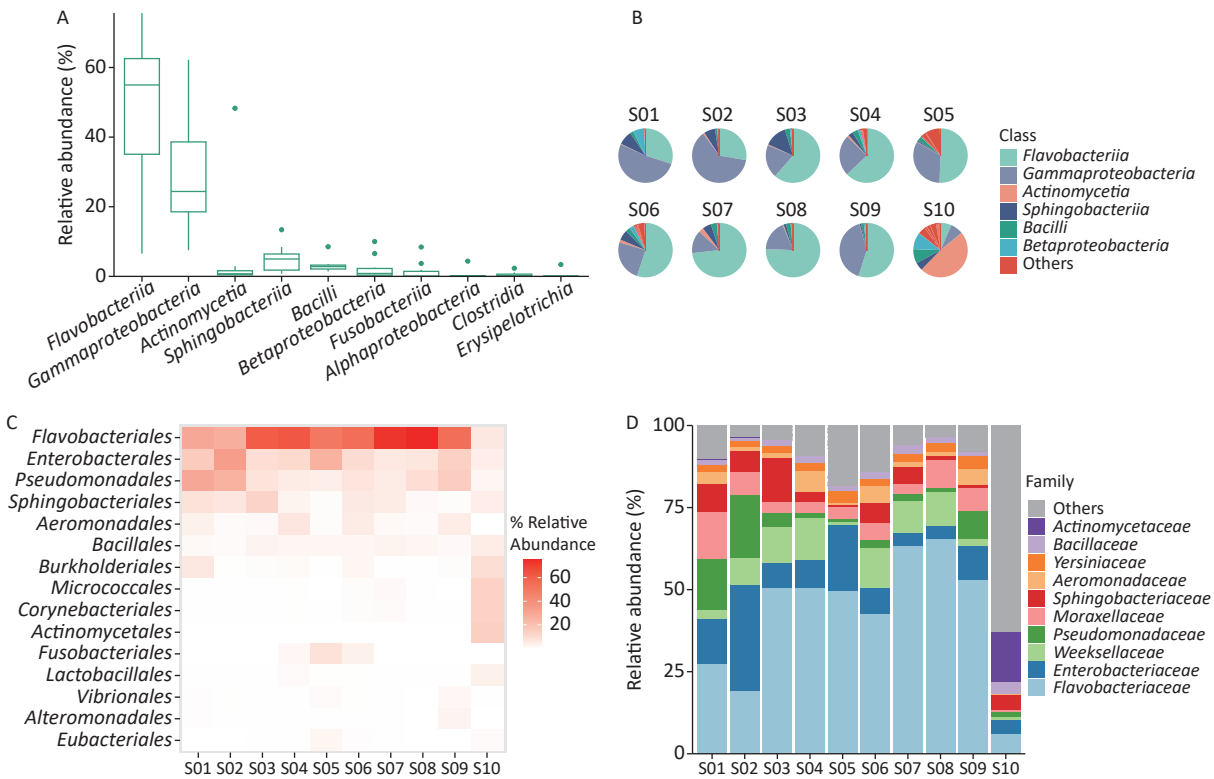

Figure 2. Identification of bacterial microbiome in environmental samples taken at Poyang Lake Animal Rescue Center using Kraken2 and Bracken. (A) The top 10 taxa at the class level of total relative abundance of identified bacterial sequence reads. The composition and diversity of bacterial microbiome identified in ten samples of (B) the group-mean pie plot of the top 6 taxa at the class level, (C) heatmap of the top 15 taxa at the order level and (D) bar plot of the top 10 taxa at the family level.

and *Caudovirales* were from the phyla *Pisuviricota* (only in S10) and *Uroviricota* (S1–S10), respectively (Figure 3A, Supplementary Table S1). Six virus families, *Autographiviridae*, *Chaseviridae*, *Herelleviridae*, *Myoviridae*, *Picornaviridae*, and *Siphoviridae*, were involved; only the family *Picornaviridae* belonged to the order *Picornavirales*. *Myoviridae* was the most abundant family (47.29 %), followed by *Herelleviridae* (28.87%) according to the taxonomic analysis results. (Figure 3A and Supplementary Figure S3).

The proportion of *Picornaviridae* in S10's virome was 98.11%, while there were no *Picornaviridae* in the other samples (Figure 3B). *Megrivurus* was the most abundant genus in S10, and was only found in S10 (Figure 3C). Unclassified species were observed in S01, S05, and S09 (Figure 3D).

We used the Shannon and Simpson indices to illustrate viral diversity based on the Bracken output (Supplementary Figure S2D–F). S10 had the lowest Shannon and Simpson index values of all samples. PCoA analysis showed that S10 were far removed from the other samples in the viral component.

Novel Positive-Sense Single-Strand RNA Viruses of Megrivirus

Picornaviridae is a small, non-enveloped, single-stranded RNA virus that infects a wide range of hosts. We found *Megrivirus* in S10 and assembled a full-length genome of 9,184 nucleotides.

After mapping of the trimmed reads, 99,110 reads were mapped with 100% coverage and an average depth of 809.37-fold. This genome includes an open reading frame (ORF) of 8,124 nt and encodes 2,707 amino acids. The 5'UTR contained a highly conserved nucleotide motif 'TGGTGCTGAAATATTGCAAG' (with unknown function), which was also observed in *Picornaviridae* of avian origin, including turkey hepatitis virus (genus *Megrivirus*), duck hepatitis A virus-1 (genus *Avihepatovirus*), quail picornavirus (unassigned genus), pigeon picornavirus B (unassigned genus), and *Anativirus* (genus *Anativirus*)[18-21]. The BLAST result of the ORF showed the best hit to the polyprotein of pigeon mesivirus 2 (AGS15016.1), which belongs to *Mesivirus* with 84.10% amino acid

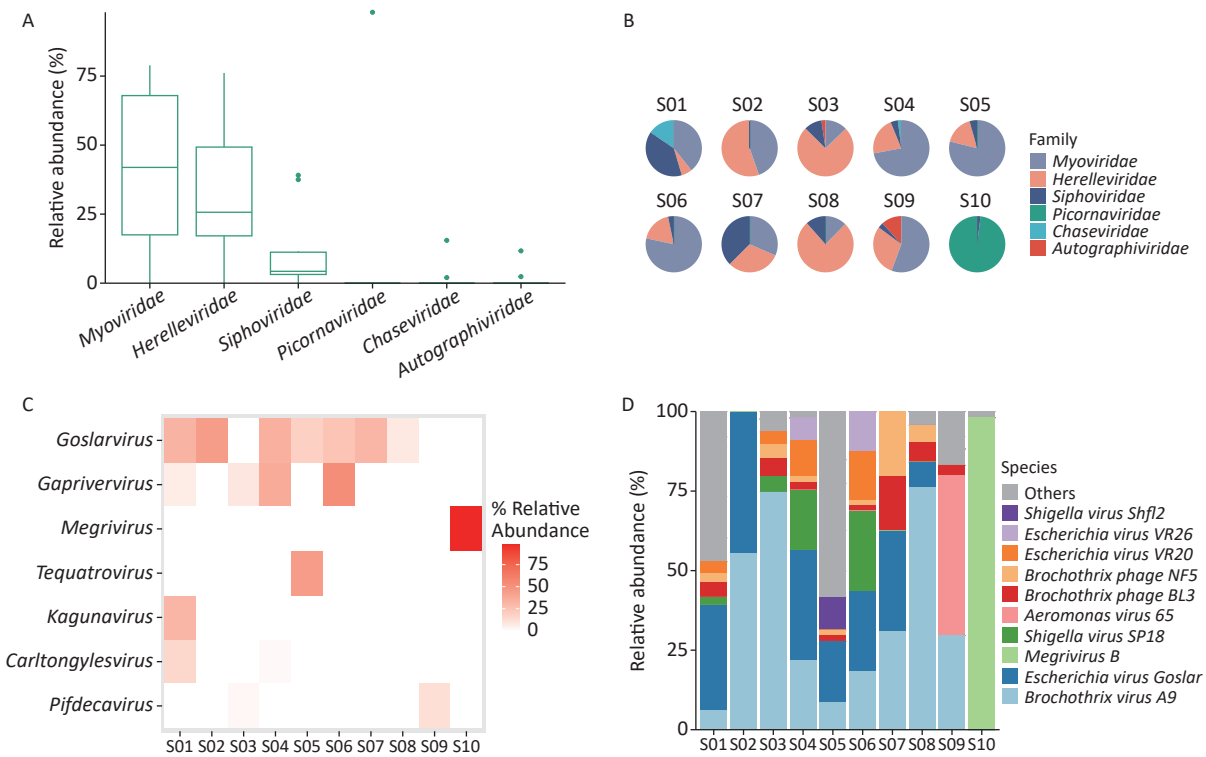

Figure 3. Identification of viruses in environmental samples taken at Poyang Lake Duchang Tangkou Wildlife Rescue Station using Kraken2 and Bracken. (A) The top 6 taxa at the family level of total relative abundance of identified virus sequence reads. The composition and diversity of viruses identified in the ten samples of (B) the group-mean pie plot of the top 6 taxa at the family level, (C) heatmap of the top 6 taxa at the genus level, and (D) bar plot of the top 10 taxa at the species level.

identity. This virus was named NC-BM-233 pigeon mesivirus-like virus (NC-BM-233). The hypothetical cleavage map of the polyproteins of *Mesiviruses* was derived from alignments with other known picornaviruses. However, we could not determine the potential cleavage site of VP1/2A1 (Table 2).

Based on the amino acid sequence of RdRp, phylogenetic analysis showed that NC-BM-233 clustered with viruses from pigeons (Figure 4). Sequences of NC-BM-233 and pigeon mesivirus clustered into *Megrivirus B*.

In addition, we found that 36 of the 39 host contigs in S10 belonged to the family *Columbidae*. This result was consistent with that of the caged turtle dove host.

DISCUSSION

The ecology of waterfowl is rich in microorganisms and viruses, which may cause cross-species transmission[22]. Poyang Lake is an important relay station for migratory birds, with many migratory birds stopping during the annual migration season. A few birds were injured for various reasons and could not survive independently. Migratory birds in need of rescue in the Poyang Lake District were rescued by volunteers and taken to the rescue station for treatment. There were many injured migratory birds in the limited space of the rescue station, which could have increased the risk of pathogen transmission. Therefore, it is particularly important to understand pathogenic pathogens by analyzing the diversity of bacteria and viruses in the living environment of birds at animal rescue stations. In this study, we used ten surface-smearing samples from rescue station cages to perform a metagenomic analysis of the high diversity of microorganisms and viruses.

Among the ten samples, the bacterial microbiome analysis results showed that the class *Flavobacteriia* was the most abundant, followed by *Gammaproteobacteria* and *Actinomycetia*. The proportion of class *Actinomycetia* in S10 was much higher than that in samples S01–S09, which mainly comprised *Gammaproteobacteria* and *Flavobacteriia*. Bacterial microbiome analysis results showed that the abundance of bacteria in cage-smearing samples was inconsistent with that of the Poyang Lake water body at the class level[22].

The class *Actinomycetia* is one of the largest lineages in the domain *Bacteria*. *Actinomycetaceae*, *Microbacteriacea*, *Corynebacteriaceae,* and *Mycobacteriaceae* were the top four of the most abundant families of *Actinomycetia* in S10. They were also the common families in avian-associated metagenomes[23,24]. A greater abundance of the family *Actinomycetaceae* was associated with obesity in humans[25]. However, the samples in this study were collected from the surface of the cages, and no host weight data were available. *Microbacteriacea* is a gram-positive bacteria common in bird droppings and soil environments[24,26]. *Corynebacteriaceae* and *Mycobacteriaceae* are collectively known as Corynebacterium-Mycobacterium-Nocardia (CMN) bacteria. The cell walls of CMN bacteria contain

Table 2. Coding potential/putative proteins of the genome of NC-BM-233_pigeon_mesivirus_like

Putative protein	Proteinase	Location	Length (aa)
P1	VP0	M1-Q390	390
	VP3	T391-Q558	168
	VP1	G559-D807[1]	249
P2	2A1	E808[1]-S1097	290
	2A2	R1098-E1290	193
	2B	A1291-E1480	190
	2C	A1481-E1826	346
P3	3A	S1827-E2008	182
	3B	A2009-E2036	28
	3C	G2037-Q2233	197
	3D	G2234-L2707	474

Note. [1]The cleavage site of VP1/2A1 has not been fully predicted.

mycolic acid, which renders them less susceptible to a wide variety of antimicrobials[23].

The class *Flavobacteria* widely exists in fresh water, seawater, soil, and plants[22,27]. *Gammaproteobacteria* in S01–S09 included *Salmonella enterica*, *Pseudomonas aeruginosa*, *Yersinia pestis* and *Acinetobacter baumannii*. *Salmonella enterica* is a type of intestinal bacteria, which is often caused by ingesting unclean food, leading to severe diarrhea in infected individuals, and is one of the main pathogens of human food poisoning[28]. *Salmonella pullorum* mainly affects young chickens and causes septicemia and mass death. *Salmonella pullorum* causes brooding nest inflammation in adult hens and infected adult hens, carrying bacteria in the yolk and passing it to the chicks[29]. *Pseudomonas aeruginosa* is widely distributed in natural and normal skin, intestinal tract, and respiratory tract and is a common opportunistic pathogen in clinical practice[30]. *Yersinia pestis* is a vector of bubonic, pneumonic, and septicemic plague. *Acinetobacter baumannii* is a common nosocomial infection and pathogen in aquaculture animals, and it usually leads to bacteremia, pneumonia, meningitis, peritonitis, endocarditis, urinary tract, and skin infections[31].

The virus analysis results showed that *Myoviridae*, *Herelleviridae,* and *Siphoviridae* families were the dominant virus families of S01–S09, which is similar to the virome components of a freshwater Amazonian lake at the family level[32]. *Myoviridae* and *Siphoviridae* can infect *Cyanobacteria*, which are also known as cyanophages and are well-known phage families prevalent in multiple poultry feces[33]. An imbalance in phage diversity and abundance can lead to changes in poultry ecosystems.

The dominant virus family in S10 was *Picornaviridae*. *Picornaviridae* is a single-stranded RNA virus that is common in avians and can infect many types of avian-causing duck viral hepatitis, turkey viral hepatitis, and avian encephalomyelitis[18,34-39]. In S10, *Megrivirus* is the

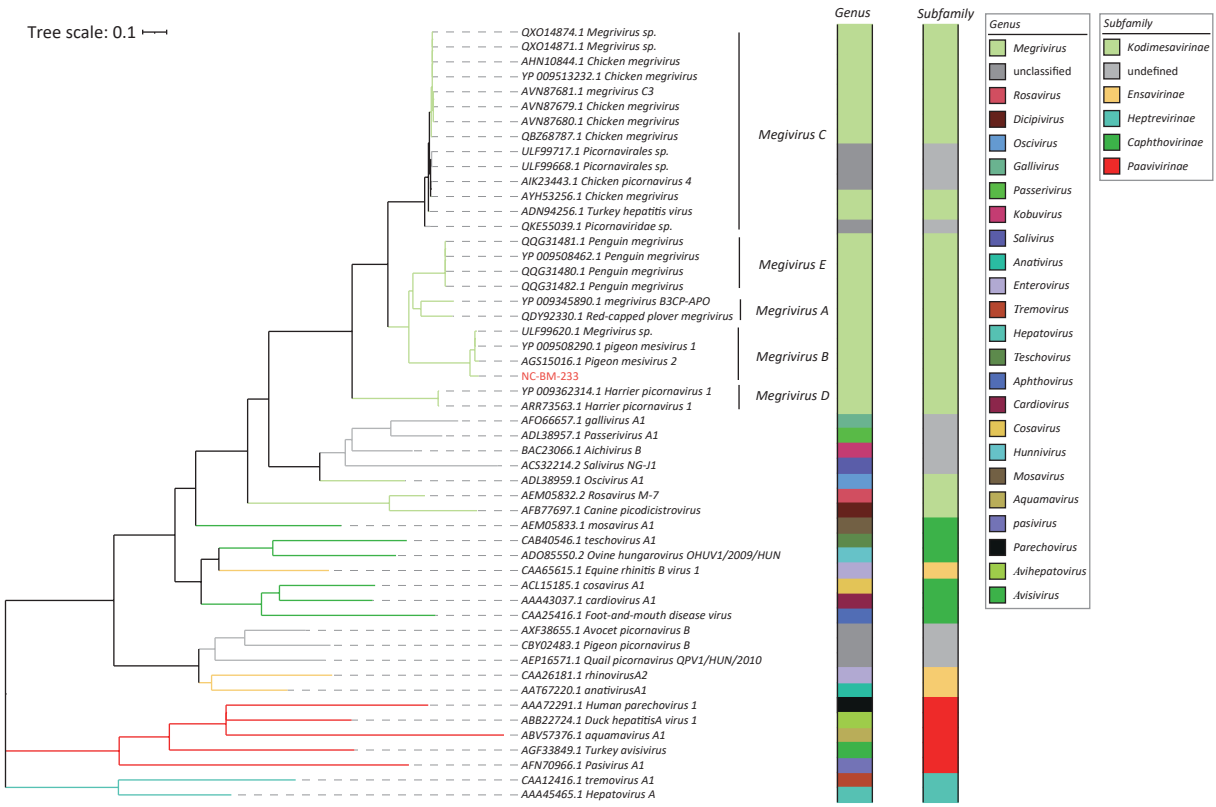

Figure 4. Phylogenetic analysis of viruses RdRps belonging to family *Picornaviridae*. The phylogenetic tree was constructed using the maximum-likelihood method with 1,000 bootstrap replicates. The branch widths are calculated from the bootstrap values. The virus sequence identified in this study is showed in red. Taxon information of genus and subfamily is shown as colored strips. The branch color was consistent with the subfamily information.

main component of the family *Picornaviridae*, which is widespread in healthy and diseased chickens and could lead to infectious viral gastritis in chickens[36]. We identified a new virus belonging to *Megrivirus B*, named NC-BM-233. A highly conserved 20 nucleotide motif was common in small RNA viruses of avian origin[18-20]. The genus *Megrivirus* can infect many avian species and cause diseases, indicating a potential threat to wild birds. Moreover, wild birds at Poyang Lake migrate hundreds of kilometers from Siberia to Australia, visiting many water bodies along the migration route, which enhances the chance of infection and transmission of multiple regions and hosts.

We must admit that the data results based on these reads have limitations. It is easy to misjudge the reads of low-abundance species in a taxon analysis, which may lead to inaccurate results. As metagenomics is developing rapidly, new classification technologies may provide new knowledge from our data in the future. The virome and bacterial microbiomes of S10 were different from those of the rest of the samples in this study, which might be caused by the presence of the virus NC-BM-233. Whether there is a correlation between the virome and the bacterial microbiome is worthy of further study. The hydrolysis site of VP1/2A1 in NC-BM-233 also needs further investigation.

In summary, these results indicate diverse bacterial microbiomes and viral communities in the Poyang Lake wildlife rescue station and provide a fresh perspective on the bacterial microbiome and virome diversity related to birds, and a new viral genome was discovered. Continuing to investigate bird-relative samples at Poyang Lake will provide more information about the microbiome and virome, especially for discovering potential pathogens.

AUTHOR CONTRIBUTIONS

JL, LY, and DW designed the study. JL, XZ, XL, WS, and HL compiled and curated the data, performed bioinformatics analysis, and interpreted the results. DW and YL supervised and administered the project and provided funding. JL, LY, and DW wrote the original draft and manuscript with inputs from XZ, XL, WS, and HL. All authors critically reviewed and approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in the following online repositories: https://www.ncbi.nlm.nih.gov/; https://www.ncbi.nlm.nih.gov/sra/PRJNA862611; https://www.ncbi.nlm.nih.gov/nuccore/OP169446.

CONFLICTS OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

Received: November 28, 2022;
Accepted: February 15, 2023

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