



OPEN Identification of a newly discovered virus from *Culex* and *Armigeres* mosquitoes in China

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Mosquito associated virus have always been a significant threat to global health. Metagenomics offers a straightforward and quantitative means to acquire the information of novel virus and has greatly enriched the content of mosquito associated virus databases. During an entomological surveillance for arthropod-borne viruses in China, we identified a previously unrecognized virus from mosquitoes, temporarily named Huajieling virus. In this study, a total of 3,960 mosquitoes were collected and then divided into 91 pools, according to location and species. QRT-PCR and nested PCR were performed to confirm the presence of Huajieling virus. Its genomic features and phylogenetic relationships were further analyzed. Our results showed that Huajieling virus was detected in 7 of the 91 mosquito pools and that the minimum infection rate (MIR) was 0.18% (7/3,960). One complete genome sequence and 2 viral partial sequences were obtained from the Huajieling virus-positive pools. Pairwise distances analysis indicated that these amplified sequences shared high nucleotide identity. Phylogenetic analysis demonstrated that Huajieling virus is most closely related to Wufeng shrew picorna-like virus 43, which belonging to *Picornavirales*. Further analyses indicated that Huajieling virus is a new member of unclassified *Picornavirales*, and is intermediate between the family *Caliciviridae* and *Secoviridae* in taxonomic status.

Keywords Huajieling virus, Mosquitoes, Genome, Evolution, Shandong Province

Arboviruses are a large group of viruses that cause natural epidemics through the bite of blood-sucking arthropods on sensitive vertebrates. Common vectors include mosquitoes, ticks, midges, flies, mites, and sandflies, among which mosquitoes are considered the most important vector of arboviruses¹. Currently, over 600 species of arbovirus have been discovered worldwide, of which approximately 130 can cause diseases in humans and animals. There are over 300 species of arbovirus transmitted by mosquitoes, accounting for more than 50% of the total, widely distributed in all continental regions except the South and North Poles². It has been found that the mosquito-borne viruses that are most closely related to humans belong to the Alphavirus, Flavivirus and Bunyavirus, such as Japanese encephalitis virus, dengue virus, Zika virus, Yellow fever virus, West Nile virus, Chikungunya virus, etc., which pose significant risks to human health and life safety^{3–8}. The main clinical symptoms of human infection with mosquito-borne viruses are fever, rash, arthralgia, encephalitis and even death, and there is no specific vaccine for most mosquito-borne viruses^{9,10}. With global climate change and the development of business, trade, tourism, and transportation among countries, many mosquito-borne viruses have mutated to produce new virulent strains in tropical regions, causing outbreaks and epidemics in temperate regions. In addition, new mosquito-borne viruses are constantly being discovered, making their threat increasingly serious worldwide^{11–15}. As a result, mosquito-borne viruses and their associated diseases have become a hot public health issue of concern to the international community.

Shandong Province is located on the coast of East China, adjacent to the Bohai Sea and Yellow Sea spanning between 34°22.9'–38°24.01'N, and 114°47.5'–122°42.3'E. The terrain is mainly plain and hilly, with the central mountainous area protruding and the western low-lying and flat area. The area spans the five major water systems of the Huai River, Yellow River, Hai River, Xiaoqing River, and Jiaodong River. It belongs to a warm temperate monsoon climate, with concentrated precipitation and rainy and hot seasons. The winter and summer seasons are longer, and the average annual precipitation is 676.5 millimeters¹⁶. Shandong Province is rich in biological resources, with a wide variety of terrestrial invertebrates, especially insects, ranking first among similar species in the country¹⁷. Among them, mosquito species are relatively diverse, with densities peaking during the summer

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months^{18,19}. In this study, a new virus, tentatively named Huajieling Virus, was discovered by collecting mosquito specimens and identifying the virus in August 2022 in the rural area of Yantai City, Shandong Province, China. Whole genome sequence analysis showed that the virus was relatively close to Wufeng shrew picorna-like virus 43 in terms of gene homology, and it was hypothesised that it might be a variant of this virus or more closely related to it with a common evolutionary ancestor. In addition, we also conducted peripheral blood virus nucleic acid tests on nearby cattle in an attempt to reveal the life cycle of the virus. This study offers new insights into the diversity of mosquito associated virus and provides fundamental data for advancing research on mosquito associated virus-mosquito-borne viruses, as well as for developing public health prevention and control strategies in Shandong.

Methods and materials

Mosquito specimen collection

In August 2022, we collected mosquitoes from six sampling sites in the Huajieling area of Yantai City, Shandong Province, China, using UV mosquito trapping lamps (Kongfuxiaoshua, China). The collection sites were chosen near the feeding grounds of local farmers' domesticated animals, and the collection time was from 18:00 to 6:00 the next day. The collected specimens were transferred to the laboratory as soon as possible and placed in a cool place at room temperature for 8 h in order to digest any ingested blood that might be present. After that, the mosquitoes were subjected to preliminary morphological classification under low temperature conditions²⁰. The males were discarded and the females were separated, preserved, registered and pooled according to the different species and the location of the collection. The number of female mosquitoes in each pool did not exceed 50. And the cytochrome oxidase subunit I (*COI*) gene was amplified using conventional PCR to further verify the mosquito species²¹.

Animal serum collection

Domestically raised cattle are commonly raised and widely distributed in the local area. Therefore, in this study, we chose cattle near mosquito collection sites as blood collection objects. A certain number of randomly selected yellow cattle were subjected to jugular vein blood collection, and about 5 ml of blood was collected from each animal, which was transferred to the laboratory as soon as possible, centrifuged for serum, and stored at 4°C for further use.

Nucleic acid extraction

600 µl of pre-cooled 1640 medium was added to each pool, and mosquito tissues were broken by oscillation using a Tissuelyser oscillator (QIAGEN) at a frequency of 30 times/s for 5–10 min until the tissues were broken into homogenates. The tissue was then centrifuged at 15 000×g for 30 min at 4°C, according to the manufacturer's instructions. Finally, 150 µl of post-centrifugation supernatant and cattle serum were subjected to nucleic acid extraction by the TIANamp Virus RNA Kit (Tiangen, China). The obtained RNA was immediately detected or stored at -80°C in the refrigerator.

Library construction

RNA from all mosquito pools was aspirated 2 µl each and mixed into a large assay tube for RNA library construction. Nucleic acid concentration quantification and rRNA removal were performed on the library. Specifically, samples with RNA concentrations below 10 ng/µl were not subjected to rRNA removal, while samples above 10 ng/µl were subjected to rRNA removal using the FastSelect rRNA Kit (Novozymes). The rRNA removed included cytoplasmic 28 S, 18 S, 5.8 S, and 5 S rRNA, as well as mitochondrial 16 S and 12 S rRNA. This kit retains messenger RNA (mRNA) and other non-coding RNAs, such as long non-coding RNAs (lncRNAs). Then, the NGS library was constructed using VAHTS® Universal V8 RNA-seq Library Prep Kit (Novozymes). Finally, the constructed libraries were sent to Beijing Geing Medical Laboratory Co., Ltd. for quantitative quality control by Agilent 2100 Bioanalyzer and qPCR, and the library was rolled and replicated to form nanorods and loaded onto the sequencing chip, and then subjected to high-throughput sequencing.

Sequence alignment

In this study, a DNBSEQ-T7 sequencer (BGI) was used, and the double-end sequencing method (2×150 bp) was selected for sequencing. The sequencing data of next-generation sequence (NGS) were analyzed by CLC Genomics Workbench version 23.0.2 (Qiagen) for quality control (QC) and trimming of low-quality sequencing data. Reads filtering for mosquitoes were performed using STAR v2.5.0a by mapping the host reads with host rRNAs and genome sequences. Then, the remaining nonhost reads were matched to assembled contigs through de novo method using MEGAHIT v1.1.2. As we previously described²², the assembled contigs were first aligned against viral nucleotides and proteins using blastn and blastx with an E-value cutoff of 1E-2 to identify the viral contig. Then, these contigs were compared using blastn (E-value cutoff at 1E-10) with the nonredundant nucleotides database (downloaded in May 2024) to eliminate false positives. Novel viruses were recognized as those viruses that had > 50 read counts and < 60% identity to any known virus.

Detection of Huajieling virus RNA in mosquitoes and sera

QRT-PCR was performed on QuantStudio™5 Real-Time PCR system (ThermoFisher Scientific, USA) for the detection of the Huajieling virus from mosquito pools and nested PCR was applied to amplify positive samples to obtain sequence information. In this study, the one-step RT-PCR method was utilized and virus-specific primer-probe sets were described as follows: F(GCA CAA AAG TCC AGC GAA GAT), R (CTG TAT CGC TTC AAG AAC TCC AAC T), P (CAC CGT TCG ACC GCT TGC CG). The reaction was carried out in a volume of 25 µl, that contained 5 µl 5×buffer, 1 µl dNTP, 1 µl enzyme mix, 11.75 µl RNase-free water, 0.5 µl upstream

primer and downstream primer, 0.25 μ l probe, and 5 μ l RNA. The qRT-PCR was performed at 50°C for 30 min and 95°C for 15 min, and this was followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 10 min. The cycle threshold (Ct) value for a positive sample was set at 35 cycles. In addition, all positive pools by qRT-PCR were further validated by nested PCR using primer sets: Out-F (GCA AGC ACA GAT TGG CGA T), Out-R (CGT CGT CTT GAT CCG TTC GT), In-F (TCG AAG ATC TGG CCG TGA GT) and In-R (GTG AGC TTT GAA CGC CGT GT). The reaction was carried out in a volume of 20 μ l, containing 10 μ l 2 $\times$ Buffer, 6 μ l RNase-free water, 1 μ l upstream primer and downstream primer, and 2 μ l templates. The nested PCR reaction conditions were 98°C for 10 s, 55°C for 5 s, 72°C for 2min10sec and 98°C for 10 s, 55°C for 5 s, 72°C for 1 min 20 s, with the reaction lasted for 40 cycles. The amplified products were separated by electrophoresis in a 1% agarose gel electrophoresis, and the electrophoresis products were then visualized by SYBR[®] Safe (Thermo Fisher Scientific) and subjected to direct Sanger sequencing (Shanghai Sangon Biotechnology Co., Shanghai, China).

Virus isolation

Specimens positive for Huajieling virus nucleic acid were selected for virus isolation using Baby Hamster Kidney 21 (BHK-21) and *Aedes albopictus* clone C6/36 (C6/36) cells. The mosquito homogenate was centrifuged to obtain the supernatant and passed through a 0.45 μ m sterile filter (MilliporeSigma, Burlington, MA, USA). Then, 50 μ l of the filtrate was taken and inoculated onto approximately 70% fused monolayers of cells in 24-well culture plates and routinely incubated for 1 h. The supernatant was then discarded, 1 ml of cell maintenance solution was added, and the cells were placed in an incubator and continued to be cultured for 10–12 days, and the cytopathic effects (CPE) were observed under a microscope every 12 h. All specimens were blindly passed for 3 generations. Virus isolation was performed at a BSL-2 laboratory in the School of Public Health, Shandong Second Medical University.

Data analyses

The prevalence of Huajieling virus in mosquitoes was calculated as follows: minimum infection rate (MIR)²³, = the number of positive pools / the total number of mosquitoes tested. The statistics analyses were calculated and performed using Prism software version 5.00. Fisher's exact test was performed to evaluate the statistical difference in the positive rate. Differences with $p < 0.05$ were considered to be statistically significant.

The complete nucleotide sequence of Huajieling virus was analyzed using NCBI's ORF finder tool to identify the open reading frame. The available sequences of related viruses were obtained from the GenBank database via BLASTN, and they were compared with Huajieling virus obtained in this study. Protein model prediction was conducted with the Swiss-Model software, subcellular localisation prediction of proteins was performed with the Cell-PLoc 2.0 tool, and signal peptide prediction was carried out with the Novo Pro software. Meanwhile, the complete nucleotide sequence was deposited in GenBank under the accession number PP097215.

Results

Mosquito collection and viral nucleic acid detection

A total of 3,960 female mosquitoes were collected from livestock shed habitats at six sampling sites in Yantai City, Shandong Province (Fig. 1). After morphological identification, these mosquitoes belonged to 3 genera and 3 species, namely *Culex quinquefasciatus*, *Armigeres subalbatus*, and *Anopheles sinensis* (Table 1). Among them, *Culex quinquefasciatus* was the dominant mosquito species in livestock sheds with 3544 mosquitoes, accounting for 89.49% of the total number of captures. *Armigeres subalbatus* and *Anopheles sinensis* accounted for a lesser proportion of 9.09% and 1.41%, respectively. Based on the different collection locations and species, we classified all mosquito specimens into 91 pools, of which 7 pools tested positive for the Huajieling virus nucleic acid, involving two species of mosquitoes, namely *Culex quinquefasciatus* and *Armigeres subalbatus*. The cycle threshold (Ct) values for the virus-positive samples ranged from 17.04 to 34.25, with a mean of 29.66 and a standard deviation of 2.77. The MIRs for these two mosquitoes were 0.17% and 0.28%, respectively, but the differences in positive rates between mosquito species were not statistically significant.

Genome-wide analysis of Huajieling virus

In total, the library generated 9.72G base (Gb) of data including 32.4 million or so 150 bp paired-end reads. After removing host sequences, we achieved 6088 clean reads related to Huajieling virus with the average coverage depth of 82 times. The complete genome of Huajieling virus was successfully obtained from a qRT-PCR-positive specimen (CG13). It consists of a single-stranded positive-sense RNA with a total length of 11,108 nt, containing only one open reading frame from 187 to 8376, encoding 2729 amino acids. Conserved Domain Search showed that the ORF of Huajieling virus probably encodes only three proteins, including 2OG-Fe (II) oxygenase superfamily at amino acids 1209–2344, RdRP protein at amino acids 1744–2149 and Calicivirus coat protein at amino acids 2209–2433 (Fig. 2). However, whether these three proteins are expressed independently or are cleaved from a single polyprotein needs to be verified by further studies. In addition, by comparing with the sequences obtained from NCBI, the two representative viral proteins encoded by Huajieling virus, RdRP and Calicivirus coat protein, show the highest homology with the related proteins of Wufeng shrew picorna like virus 43, with similarities of only 43% and 38%. Therefore, Huajieling virus is likely to be a newly discovered mosquito-borne virus (Figs. 3 and 4).

Structural analysis of virus encoded proteins

Subsequent structure prediction analyses of the three proteins were conducted using the Swiss-Model selective homology modelling approach. The structure prediction model of RdRp, an indispensable enzyme in viral replication, exhibits a typical right-handed helical conformation. The structure is comprised of multiple helices

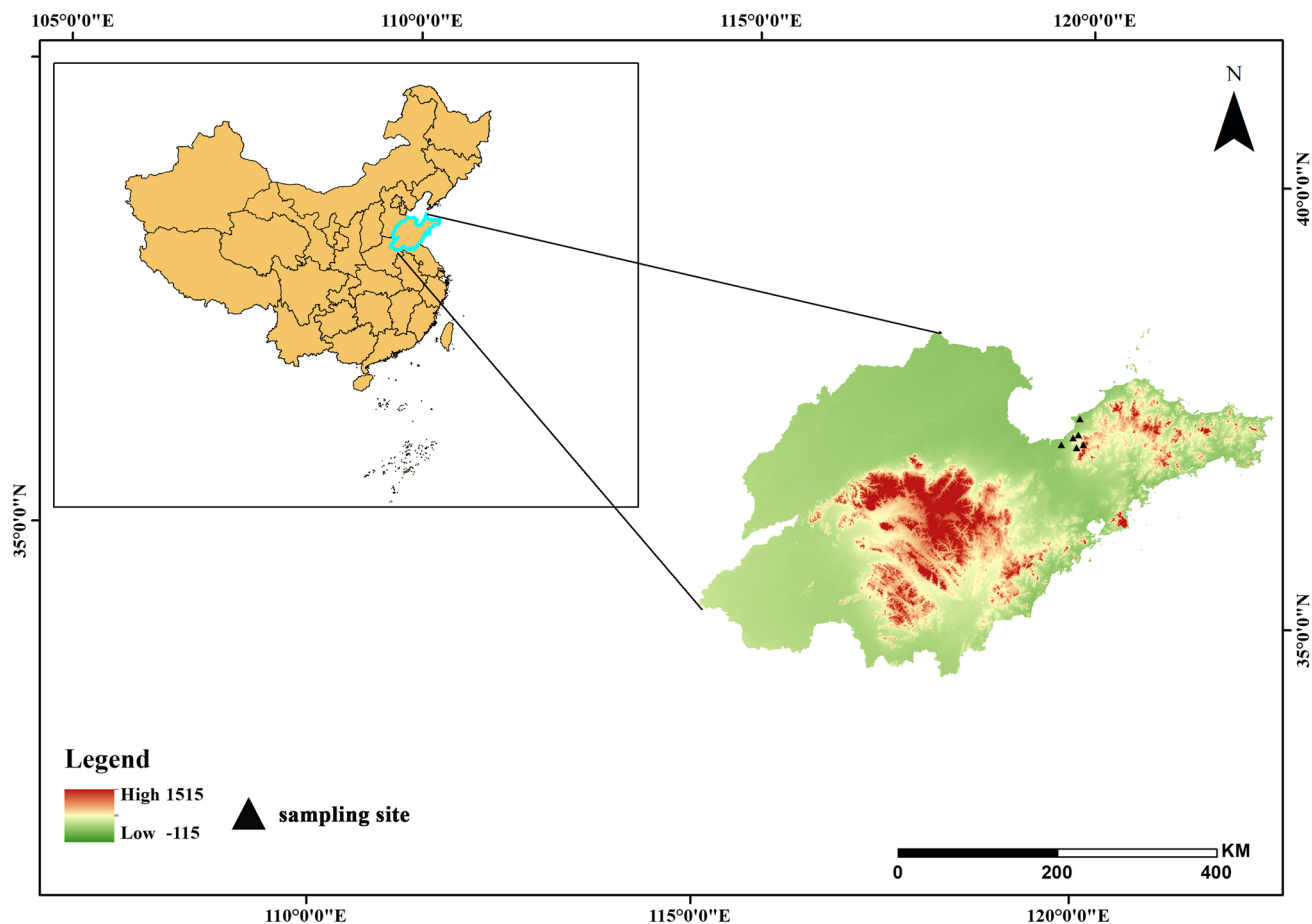


Fig. 1. Location of Yantai city, within the Shandong province where mosquito samples were collected.

Species	No. of mosquitoes	No. of pools (30–50 mosquitoes/pools)	No. of Positive pools	MIR	P
<i>Culex quinquefasciatus</i>	3544	80	6	0.17	> 0.05
<i>Armigeres subalbatus</i>	360	9	1	0.28	
<i>Anopheles sinensis</i>	56	2	0	0	
Total	3960	91	7	0.18	

Table 1. qRT-PCR to determine Huajieling virus in mosquito samples of Yantai, Shandong Province, in 2022.

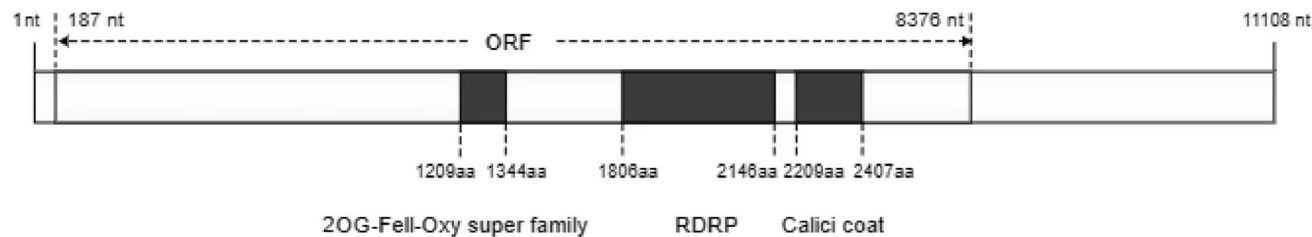


Fig. 2. Schematic representation of the genome organization of Huajieling virus.

and laminar domains, including the palm, thumb, and fingers. The bottom of the palm region and the thumb region are of particular importance for the catalysis of RNA synthesis. Subcellular localisation predictions indicate that the protein may be distributed in the cytoplasm or the nucleus. As the most conserved protein of RNA viruses, its association with host adaptation of viruses represents a significant area of research. The B-C

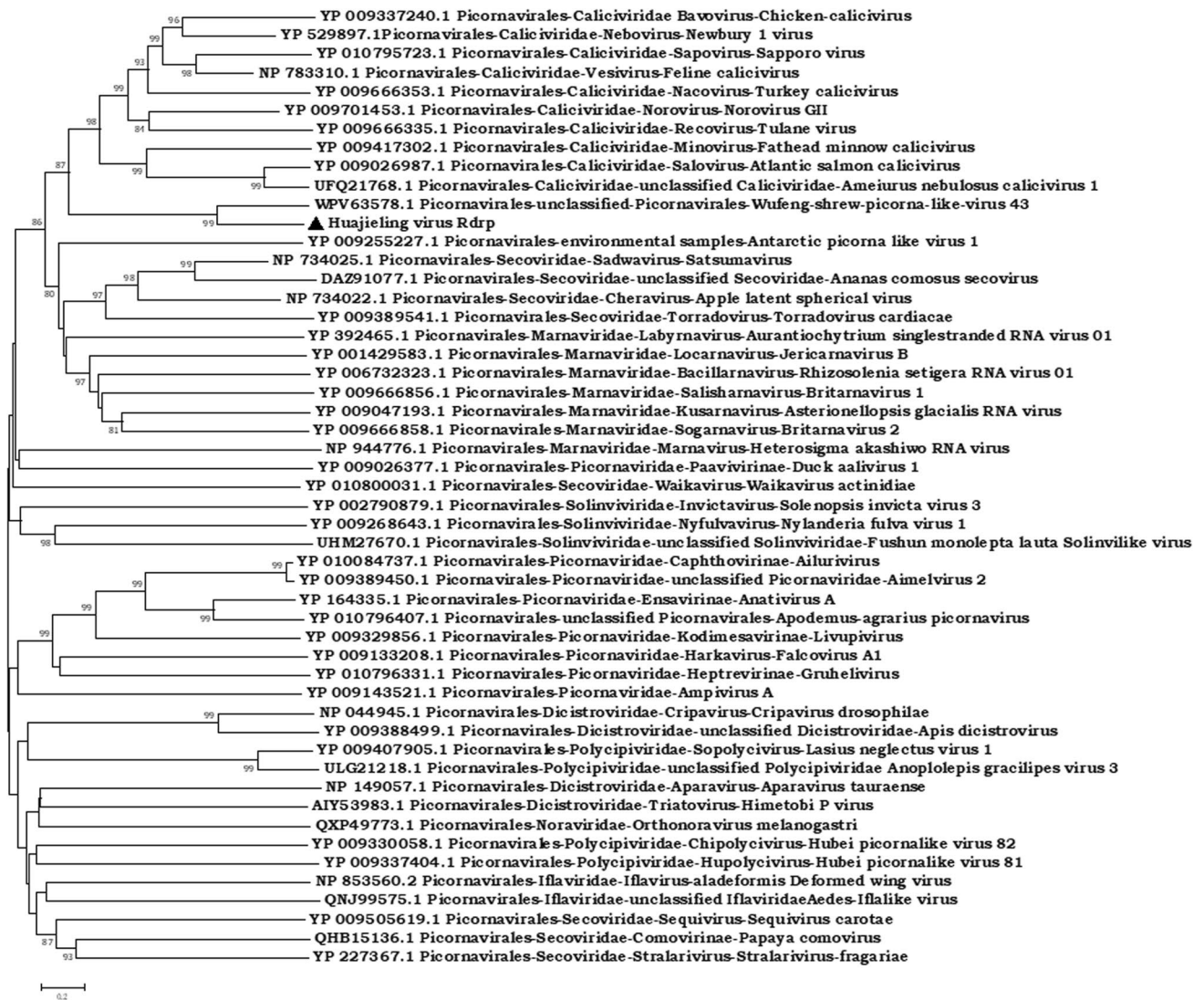


Fig. 3. Phylogenetic analysis of Calicivirus coat protein and RDRP of Huajieling virus and other representative family members, within the order *Picornavirales*. The sequences identified from mosquitoes in the current study are marked black with black triangle. Phylogenetic trees were constructed by the Maximal likelihood method using MEGA 5.1, with 1,000 replicates with bootstrap values > 70% considered significant. The numbers above the branches indicate bootstrap values.

junction region exhibits high diversity among the large family of RNA viruses, suggesting that it may represent a host adaptation region shared by RNA viruses (Fig. 5).

The calicivirus capsid protein plays an essential role in the viral particle, serving to safeguard the viral genetic material from damage caused by external factors. The structural prediction models indicate that the capsid protein comprises multiple functional domains, including an outer domain that may contain glycosylation sites, a transmembrane anchoring domain, and a cytoplasmic tail. These domains are essential for the assembly of the viral particle and its release from the host cell. Subcellular localisation predictions indicate that the protein is predominantly distributed in the cytoplasm. Concurrently, it displays the structural characteristics of a homotrimer, a configuration that is prevalent in numerous viruses. The formation of trimers may be a crucial factor in maintaining the stability and infectivity of virus particles. The global model quality estimation (GMQE) scores indicate that the model is of high accuracy and reliability (Fig. 6).

The 2OG-Fe (II)-dependent dioxygenase employs 2-ketoglutarate (2OG) as a co-substrate and iron ions as a co-factor for hydroxylation reactions. The results of the structural prediction modelling indicate that these proteins are highly stable, with secondary structures that are predominantly α -helices and irregular coiled-coils. This structural feature contributes to the maintenance of conformational stability and functional activity in catalytic reactions. Subcellular localisation prediction indicated that the 2OG-Fe (II) oxygenase family is predominantly distributed in the cytoplasm. Signal peptide prediction, however, suggested that it is present in secreted proteins. This suggests that the proteins encoded by our 2OG-Fe (II) oxygenase family may be secreted proteins, which could have a significant impact on the interaction between the virus and the host cell (Fig. 7).

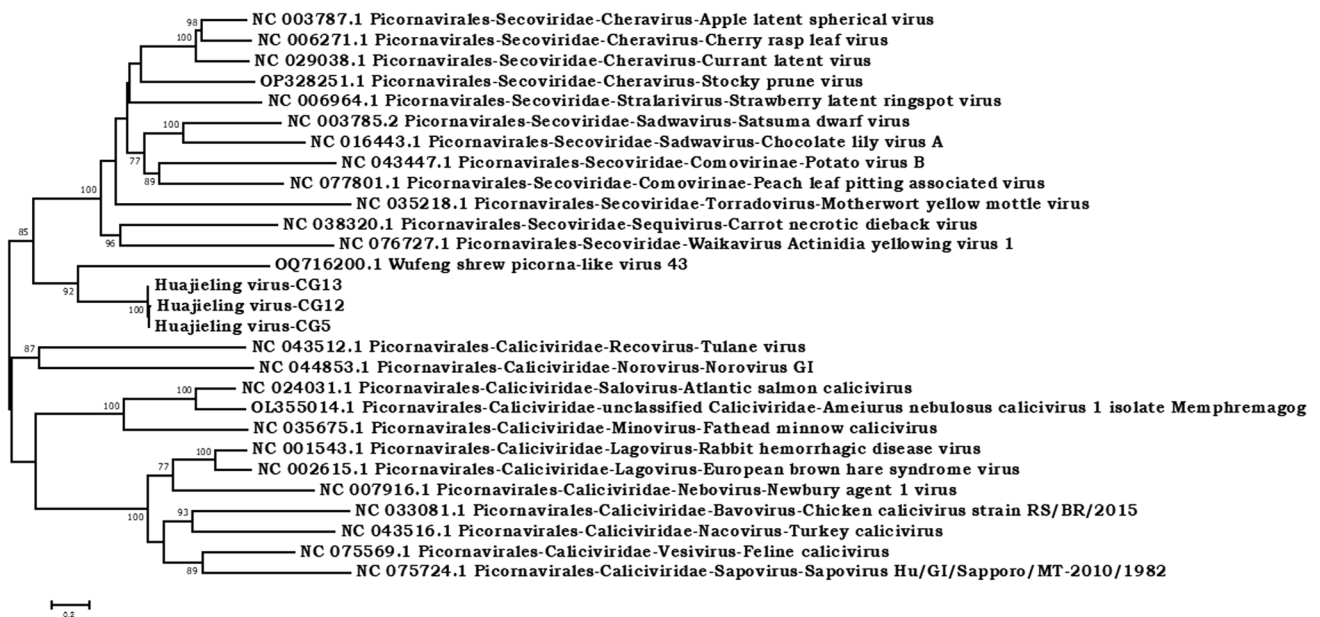


Fig. 4. Maximum-likelihood phylogenetic tree of Huajieling virus and other members of family *Caliciviridae* based on *Calicivirus* coat protein and RDRP.

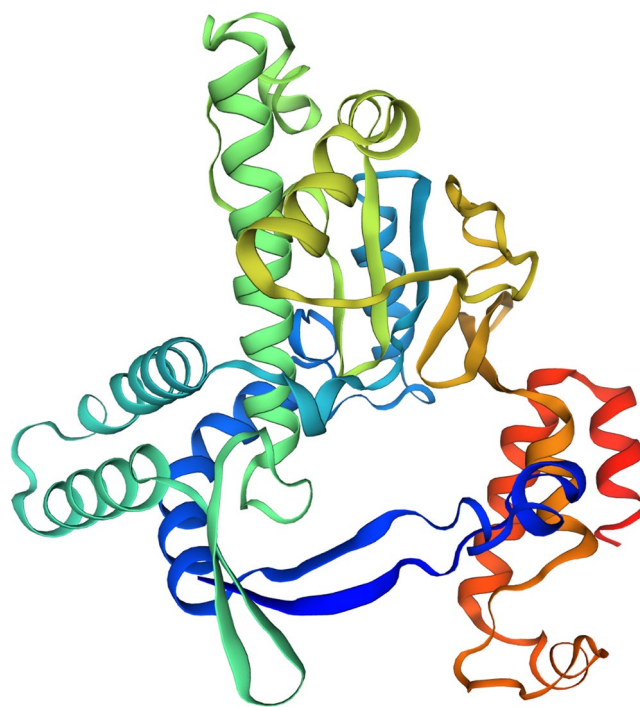


Fig. 5. 3D cartoon representation of the RdRp structure of the Huajieling virus generated by SWISS MODEL. The model includes the α -helix (Rectangles), the β -fold (Arrows), and the random coil (lines).

Nucleic acid detection in animal serum

We tested 24 cattle serum samples for Huajieling virus nucleic acid and found that three samples to be positive by qRT-PCR, with the Ct values of 32.7, 33.5 and 34.1 respectively. This result suggests that the virus may infect cattle and cause viremia, but further confirmation is needed on the viral load in cattle blood and the duration of viremia. In addition, the three positive samples came from three different sampling locations, indicating that the virus has a wide distribution range in the local area and suggesting the potential formation of a stable virus cycle.

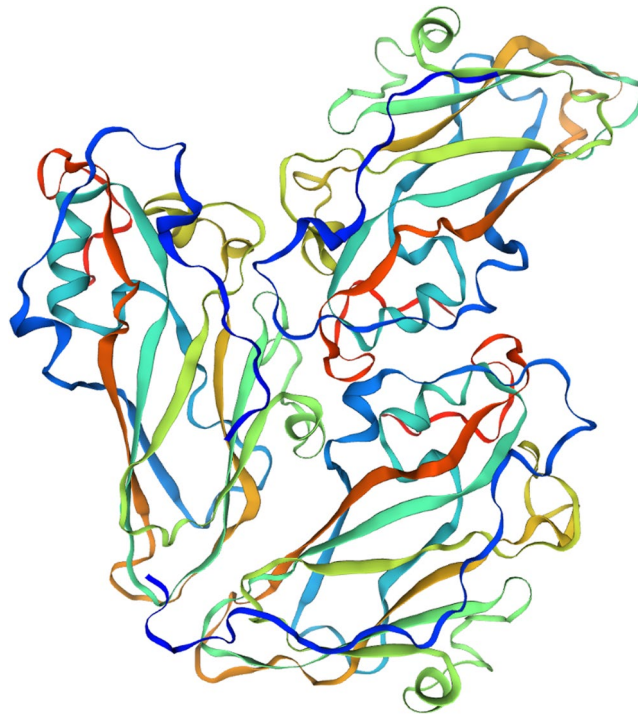


Fig. 6. 3D cartoon representation of the coat protein structure of the Huajieling virus generated by SWISS MODEL. The model includes the α -helix (Rectangles), the β -fold (Arrows), and the random coil (lines).

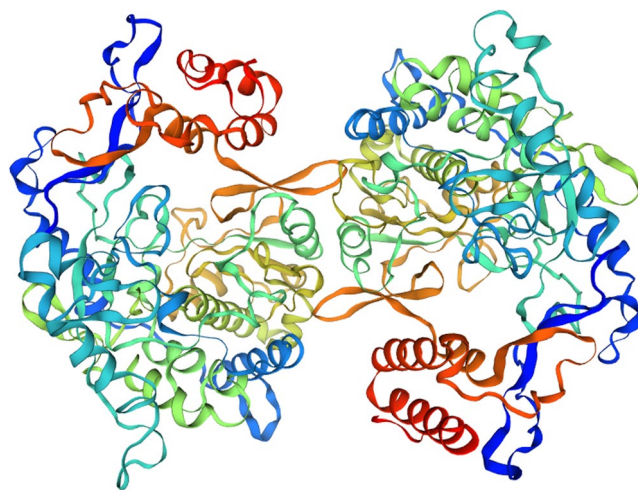


Fig. 7. 3D cartoon representation of the 2OG-FeII-Oxy super family structure of the Huajieling virus generated by SWISS MODEL. The model includes the α -helix (Rectangles), the β -fold (Arrows), and the random coil (lines).

Virus isolation

To obtain stable and passable live virus strains, we attempted to isolate the virus from all qRT-PCR positive specimens, including mosquito samples and animal serum samples. Simultaneously, the CPE of the cell monolayer was monitored and viral RNA in the cell culture supernatant was detected using qRT-PCR with Huajieling virus specific primers. Unfortunately, the virus was not isolated from any of the positive mosquito samples. Notably, after inoculation with CG13 homogenate, although neither C6/36 nor BHK21 cells showed any CPE, BHK21 cells exhibited partial cell detachment 7 days after inoculation with the first passages. In addition, the viral genome was detectable in the first two passages in both cell types, but the virus could not be traced after three passages. Collectively, neither cell line is an optimal cell for virus growth and replication. The current results do not yet provide evidence that the virus replicates in mosquito and mammalian cell lines. Consequently, in vitro purification and titration of the virus were unsuccessful.

Phylogenetic analysis

Presence of the RDRP protein is a property that is shared with members of the order *Picornavirales*, including *Secoviridae*, *Caliciviridae*, *Picornaviridae*, some unclassified viruses and so on²⁴. Moreover, among the three proteins encoded by Huajieling virus, RDRP has the highest sequence homology with reported proteins compared to the 2OG-Fe (II) oxygenase superfamily and Calici coat protein. Therefore, we chose to analyze the evolutionary relationship of the Huajieling virus by comparing the similarity of the RDRP protein. Remarkably, the top BLAST hit (i.e., highest e-value) for RDRP with relatively clear virological classification status was Wufeng shrew picorna-like virus 43, from the *Picornavirales*. Next, we explored the evolutionary relationships among the RDRP of *Picornavirales* and their homologs in Huajieling virus. After analyzing representative strains from all families and genera under *Picornavirales* by sequence comparison, we found that Huajieling virus and Wufeng shrew picorna-like virus 43 formed a monophyletic group protruded between the diversity of the family *Caliciviridae* and *Secoviridae*, suggesting that Huajieling virus is more distantly related to members of the family *Secoviridae* and belongs to a different family, while it is relatively more closely related to the family *Caliciviridae*, but still maintains a long evolutionary distance and cannot be classified as the same family. (Fig. 3). Additionally, the partial segment of Huajieling virus was amplified and sequenced from 3 of 7 qRT-PCR positive samples in this study. Pairwise distance analysis demonstrated that all sequences had nucleotide identities ranging from 97.9 to 99.0% with each other, which indicated a close relationship in evolution among these isolates. Furthermore, we also analyzed the evolutionary relationships between the three nucleic acid fragments obtained and the strains representing each genus within the families *Caliciviridae* and *Secoviridae*, and found that the Huajieling virus has a relatively independent taxonomic status and cannot be classified into any of these families. This further validates that the virus is a new virus under the order *Picornavirales*, as shown in Fig. 4.

Discussion

Arboviral infections have natural epidemiological characteristics and are mostly zoonotic diseases, making it difficult to predict epidemiological trends and posing significant risks to public health and safety²⁵. Climate change, urbanization, geographic expansion of vectors, and frequent cross-border exchanges of people and goods contribute to the rising incidence of emerging, re-emerging, and imported arboviruses, escalating the risk of arboviral epidemics^{26–28}. Therefore, it is necessary to comprehensively enhance the ability to respond to the threats and challenges posed by arboviruses, particularly by bolstering active monitoring of arbovirus, in order to clarify the virus spectrum and distribution, high-risk populations and animals, cross species transmission, as well as the genome evolution and mutation. Recently, NGS has revolutionized viral metagenomics, significantly accelerating the discovery of arbovirus and providing a powerful tool for understanding unknown arbovirus²⁹. In this research, we identified a previously unreported mosquito-borne virus, Huajieling virus, in *Culex quinquefasciatus* and *Armigeres subalbatus* from Shandong, China. After genome-wide analysis and homology analysis of the virus-encoded proteins, it was classified as a new member of the order *Picornavirales*.

By analyzing the positive rate of mosquitoes carrying viruses (MIR=0.17%, 7/3960), we can infer that Huajieling virus is widespread among local mosquitoes in Yantai City. This is also consistent with our previous research results on Hubei mosquito virus 2 (HBMV2) in the Shandong region³⁰. This suggests that the virus has adapted to the local habitat and formed a continuous and stable viral life cycle. In addition, we found the presence of Huajieling virus in two different species of mosquitoes, that the virus can spread and be transmitted through a variety of mosquito species, indicating that the vector of this virus is not singular. The virus may spread and be transmitted through a variety of mosquito species, and we cannot rule out the possibility that it can be present in other mosquito species. This also implies that Huajieling virus may have a wider distribution range and stronger transmission ability. Meanwhile, the present study found that there was no statistically significant difference in the viral carriage rates of the two mosquitoes, suggesting that Huajieling virus can survive in both mosquitoes, and that the virus does not have any obvious preference or predilection for mosquito species.

The results of animal serum virus nucleic acid testing indicate that cattle may be infected by the Huajieling virus and may serve as a natural host of the virus, but its role in virus transmission and epidemic needs further investigation to confirm. In our opinion, the next step is to conduct a prospective cohort study of cattle in the region to observe the duration of viremia in cattle and the production of related antibodies, to determine whether cattle play the role of amplification hosts in the virus cycle. Furthermore, it is worth further investigation whether the Huajieling virus can infect other mammals, and even humans.

In vitro cell separation experiments, the presence of CPE may be caused by the presence of other microorganisms in the grinding solution. Virus was not detected following three passages. The virus appeared to be simply attached to the cells and was washed away over time. Further research is required to identify the optimal cell type for the growth of Huajieling virus.

Through whole genome sequence analysis, we found that Huajieling virus is a single-stranded sense RNA virus with a small genome and simple structure, containing only one ORF. It encodes a structural protein called Calicivirus coat protein and two nonstructural proteins, 2OG Fe (II) oxygenase superfamily and RDRP proteins. After comparative analysis, we found that the similarity match between these three proteins and the currently known viral proteins is not more than 43%, indicating that Huajieling virus is likely a novel mosquito-borne virus. Our findings add a new member to the family of mosquito-borne viruses, but unraveling the full extent of the life cycle characteristics of this virus requires further research.

In terms of viral taxonomy, we attempted to localise the virus by analyzing and comparing the homology of the three proteins encoded by the virus. It was found that the 2OG-Fe (II) oxygenase superfamily was poorly classified, even in many bacteria, where the protein had a high similarity, leading to an inability to further accurately classify and localize the protein. Moreover, although the capsid protein could also be used for taxonomic positioning analysis, its small molecular weight and minimal differences among family members diminish their usefulness in precise localisation. Therefore, we selected the RdRp as the fundamental element

for classification analysis and performed phylogenetic analyses of RDRP with virus-related proteins of members within 9 families of the order *Picornavirales*. It was found that Huajieling virus clustered with Wufeng shrew picorna-like virus 43, and both sandwiched between the family *Caliciviridae* and *Secoviridae*, yet not belonging to either of them. According to existing research data, Wufeng shrew picorna-like virus 43 in diverse shrew populations inhabiting the eastern coastal regions of China. This virus was isolated from multiple shrew genera, including *Anourosorex*, *Crocidura*, *Sorex*, and *Suncus*, sampled across a wide geographic range encompassing the provinces of Shandong, Jiangsu, Zhejiang, Fujian, and Guangdong. These regions represent varied natural habitats, suggesting a broad ecological niche for Wufeng shrew picorna-like virus 43. The high sequence similarity among Huajieling virus and Wufeng shrew picorna-like virus 43 may reflect their commonalities in biological characteristics, host ranges and ecological niches. Furthermore, it suggests that these viruses share a large evolutionary homology and may have originated from a common viral ancestor. However, based on the current results of protein evolution analysis, we are unable to classify Huajieling virus accurately to a specific genus, even family, which may involve gene recombination or intermediate viruses between these two families. It is possible that with the continuous emergence of members similar to Huajieling virus, such viruses will be grouped into a new and separate family, and the present study can only provide a small clue to the virological classification of such members.

In addition, in this study, we amplified three partial nucleic acid sequences of Huajieling virus from mosquitoes at different sampling sites in this area, and their nucleotide homology exceeded 97%. This indicates that the virus strains prevalent in the local epidemic area are the same strain, and its genetic properties are relatively stable, with no significant nucleic acid sequence mutations.

Eventually, some limitations of our study cannot be ignored. First, virus-associated antibodies in animal sera were not tested to determine the status of infection in local cattle and the role they play in the viral cycle; Second, no live virus was successfully isolated. However, this result cannot be interpreted as evidence of an absence of virus and may be due to extremely low copy numbers of live virus in the sample or inappropriate cell lines.

Conclusions

In conclusion, our finding provided molecular epidemiological information for the existence of a novel virus named Huajieling virus in mosquitoes from Shandong, eastern China. And We analyzed the genome-wide characteristics of the virus and further conducted virological classification studies based on its encoded proteins, as well as describing its epidemiological profile. Next, more research is urgently needed to elucidate the pathogenicity of Huajieling virus and its natural survival cycle characteristics.

Data availability

The datasets generated and analysed during the current study are available in the [GenBank] repository, [BankIt2859361].

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Author contributions

WBZ conceived and designed the study. JHC analyzed the data and interpreted some of the results. KL carried out formal analysis and collected the specimens. HYS conducted the molecular experiments. YJL conducted a visualization of the data. LL was responsible for the supervision of the entire experiment. GYN wrote the original draft and the review. All authors have read and approved the final manuscript.

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Declarations

Ethics approval and consent to participate

All animal experiments conducted in this study were conducted in accordance with the guidelines set forth by the Animal Committee of the Second Medical University of Shandong. These guidelines ensured the welfare and ethical treatment of the participating experimental animals. The objective of this study was to ascertain whether the huajieling virus infects local mammals. This study is significant for investigating the pathogenic role of the huajieling virus in mammals. The studies were conducted on local adult yellow cattle, with a total of 24 adult yellow cattle randomly selected for blood collection at three points in the vicinity of the mosquito sampling area. No animals died during the experiment, and all experimental operations were conducted under the supervision of professional veterinarians, with a view to ensuring the welfare and humane treatment of the animals involved. The study was evaluated and approved by the Ethics Committee of Shandong Second Medical University (approval number: 2022SDL477) and conducted in accordance with the standards set forth in the ethical code of conduct. Furthermore, the procedure for collecting animal blood was licensed by the Laizhou City Animal Husbandry Bureau, thereby guaranteeing the legitimacy and ethical soundness of the study methodology. This study is performed in accordance with relevant guidelines and regulations. All methods are reported in accordance with ARRIVE guidelines.

Consent for publication

The manuscript is original and has not been published elsewhere. All authors agree to the submission and have contributed to the work. There are no conflicts of interest.

Competing interests

The authors declare no competing interests.

Additional information

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