Artykuł przeglądowy *Review*

Application of metagenomics in the animal infectious diseases*

YANLIN LI[1#], DIANGANG HAN[2#], YONG PENG[3], WEIDONG ZUO[4], BANGQUAN ZENG[5], DAI XIONG[1], JUN AI[2], JIGE XIN[1]

[1]College of Veterinary Medicine, Yunnan Agricultural University, Kunming, China
[2]Animal Quarantine Laboratory, Technology Center of Kunming Customs, Kunming, China
[3]Technology Center of Ruili Customs, Ruili, China
[4]Technology Center of Mengla Customs, Mengla, China
[5]Yunnan Provincial Center for Animal Disease Control and Prevention, Kunming, China

Received 22.10.2024 *Accepted 10.01.2025*

Li Y., Han D., Peng Y., Zuo W., Zeng B., Xiong D., Ai J., Xin J.
Application of metagenomics in the animal infectious diseases

Summary

The increasing magnitude and density of livestock production on a global scale, the progressive degradation of biodiversity, and the phenomenon of global climate change have led to unparalleled diversification and intricacy in animal diseases. Precisely predicting epidemic trends, achieving early detection of outbreaks, and accurately identifying pathogens are imperative for developing precise control strategies and mitigating the risk of epidemic dissemination. Metagenomic next-generation sequencing (mNGS) has recently transitioned from an emerging technology to a crucial instrument for the early identification and prevention of emerging infectious diseases. It also facilitates the understanding of pathogen dissemination patterns and epidemiology and holds significant promise for advancements in infectious disease management. The objective of this review is to offer guidance and support for the implementation of mNGS in animal disease diagnosis and control by consolidating achievements, emphasizing the advantages and limitations of mNGS over conventional detection methods, and clarifying its present-day usage.

Keywords: Metagenomic sequencing, advantages and disadvantages, animal disease prevention and management

As the livestock farming industry has transitioned from traditional individual practices to intensive farming methods, it faces a significant threat from epidemic diseases. Pathogens mutate and evolve, leading to the emergence of more virulent strains, which increases disease outbreaks in animals. Traditional approaches for animal disease detection mainly include pathogen identification, serological testing, and molecular-based diagnostic techniques. To some extent, these methods are restricted when it comes to detecting unculturable microorganisms under laboratory conditions. The diversity of pathogens in the environment, the majority of which remain unisolated and uncultured, necessitates further investigation. Only a small fraction of the microbial world is known. In the past, traditional detection methods have played an important role in the study of pathogens, but the limitations of these methods have become increasingly apparent with the advancement of scientific research, thus restricting the research progress in related fields. The detection of pathogens primarily involves the isolation and cultivation of microorganisms, which necessitates their viability. However, a significant number of pathogens cannot be detected in vitro, resulting in limitations such as a low pathogen positive rate, lengthy detection cycles, and reduced accuracy. Furthermore, many viruses may not show a cytopathic effect (CPE) when cultured with specific cells due to the lack of such cells for virus cultivation. Polymerase chain reaction (PCR) rapidly amplifies specific DNA sequences *in vitro*. This method was initially developed by Kary Mullis and his colleagues and exponentially amplifies minute quantities of a particular DNA fragment to millions of times its original amount within a few hours (33). Compared to other pathogen detection technologies, PCR can detect microorganisms that

* This work was supported by Key Technologies Research and Development Program of China (2022YFC2601605), the Scientific Research Project of General Administration of Customs (2022HK064), the Academician Workstation of Yunnan Province (2018IC078), the Talent Plans for Young Topnotch Talentsof Yunnan (YNWR-QNBJ-2020-154). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

# Li Y. and Han D. contributed equally to this study.

are difficult or impossible to culture in the laboratory. However, its major drawback lies in its susceptibility to false-positives caused by nucleic acid contamination. Additionally, it necessitates primer design based on known gene sequences. Notably, PCR cannot be employed to detect viruses with unknown sequences or those exhibiting significant mutations (51).

Real-time fluorescence quantitative PCR (RT-qPCR) combines the high sensitivity of PCR, the high specificity of DNA molecular hybridization, and the high accuracy of spectroscopic technology. It also possesses the advantages of relative quantification, making it more widely utilized in viral animal epidemic detection compared to conventional PCR technology. The real-time monitoring of the PCR process via fluorescence signals is a robust method for detecting and identifying pathogenic viruses (50). The results of RT-qPCR depend on the threshold cycle number (Ct value) and calibrators, and they are greatly affected by inhibitors in the sample. Sometimes, accurately detecting low-abundance samples is difficult due to high background fluorescence in the reaction system, which to some extent limits its use (52). Loop-mediated isothermal amplification (LAMP), which involves the design of four primers that target six regions of the target sequence, imposes specific requirements on the target sequence. Additionally, extensive optimization is necessary for primer sequences, concentrations, reaction temperature, and time to control the amplified fragment length within 300 bp or less. Moreover, the high sensitivity of the LAMP method results in a risk of aerosol contamination during operation that can lead to false-positive results. Furthermore, because the amplification product consists of double-stranded DNA with varying lengths and different numbers of stem-loop structures, precise quantitative analysis of reaction results is not feasible

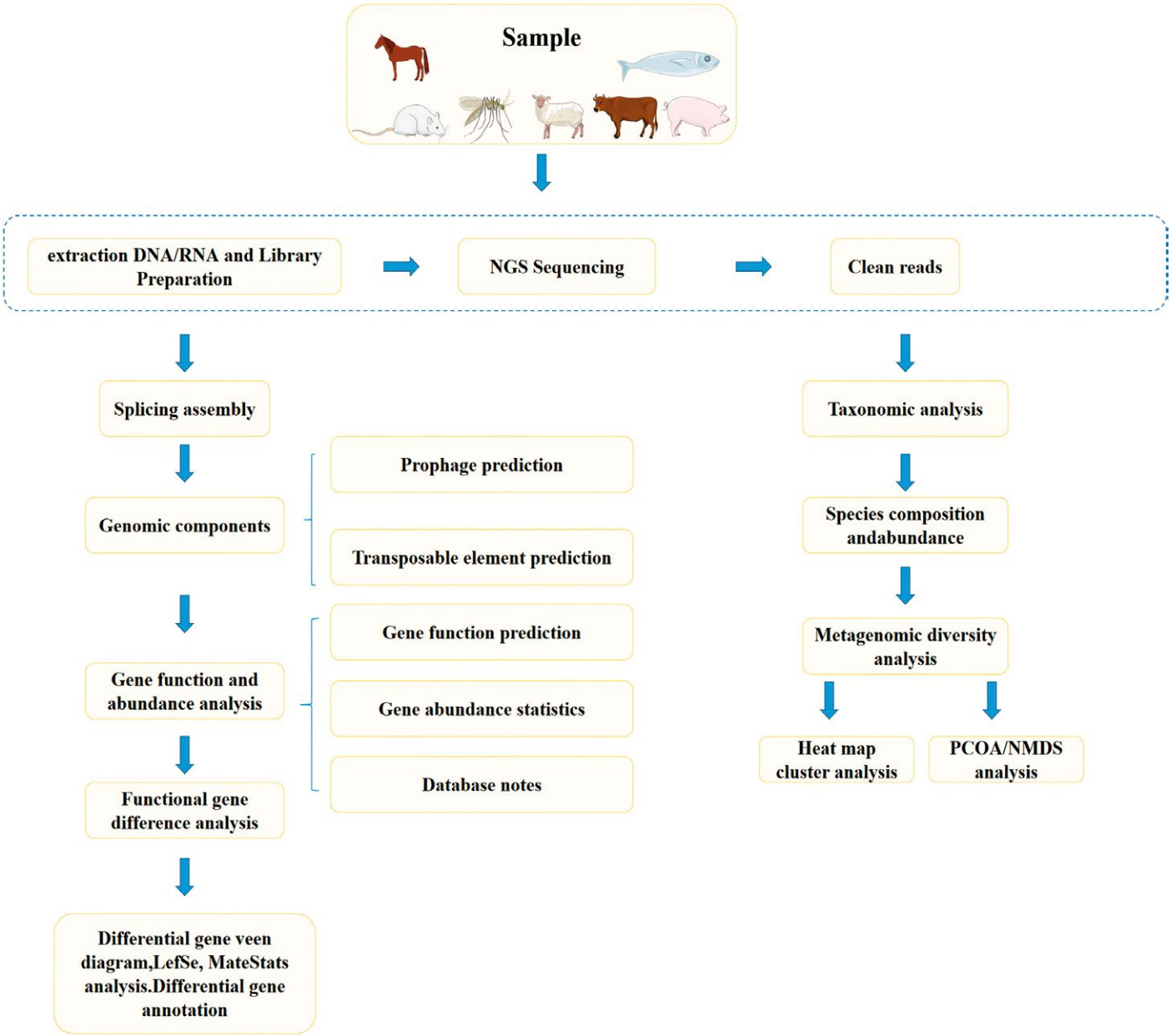

Fig. 1. Process of genomic analysis

using the LAMP method, and certain limitations exist (19). The gold immunochromatographic assay (GICA) also presents challenges in terms of limited specificity and sensitivity. Currently, the vast majority of GICA test strips applied in practical applications are only able to detect a single sample and are mostly intended for a single specific disease. Therefore, repeated sampling and testing are needed, which prolongs the time needed for disease diagnosis and increases detection costs. Antigenic drift and antigenic shift cause pathogens to mutate, thereby evading the host immune system and making pathogenic infections difficult to control. Early detection of new pathogens is therefore essential to control their spread (8).

Metagenomic next-generation sequencing (mNGS) is the analysis of the collective genomes of all members within a microbial community (39). This technique, known as mNGS, improves upon first-generation sequencing by incorporating fluorescent markers of distinct colors attached to deoxy-ribonucleoside triphosphates (dNTPs) for signal collection and processing through specialized software. Consequently, it enables the acquisition of necessary DNA information for subsequent analysis. The fluorescence variations observed during DNA polymerase-mediated synthesis of complementary chains are attributed to different types of dNTPs utilized (Fig. 1). Genomics involves the high-throughput sequencing of an organism's DNA base pairs to determine its complete genetic complement, whereas metagenomics enables the direct acquisition of any genomic sequence from the environment without requiring specific amplification. Instead, it applies high-throughput techniques to sequence nucleic acid products and detect multiple microorganisms simultaneously (24). The concept of directly cloning DNA from the environment was initially proposed by Pace (37). Schmidt et al. subsequently constructed a lambda phage library using seawater samples, in which they screened for the presence of the 16SrRNA gene (40). With the advancement of second-generation sequencing technology, metagenomic has gained extensive application in medical clinical settings for the identification of infectious disease pathogens, resistance genes, forensic identification, and epidemiological investigation. Furthermore, it has significantly reduced the time needed for clinical diagnosis and research (3). In recent years, some scholars have proposed the concept of a pathobiome based on the self-stability of microecology and microbiome in biological organisms. They believe that animal infectious diseases are caused by changes in both the quantity and quality of the entire microbiome (including bacteria, viruses, and fungi) within an ecosystem dominated by a main pathogen. Furthermore, studying pathogen gene expression and mutual metabolic relationships using mNGS in tissue systems can greatly enhance our understanding and investigation of infectious disease pathogenesis (5).

The utilization of metagenomics in the prevention and control of animal diseases

In recent years, mNGS for pathogen detection have been implemented in numerous public health laboratories, alongside standardized whole-genome sequencing (WGS) (35). The primary application areas are as follows.

Identification of novel pathogens and recurrent pathogens. Rapid pathogen detection plays a crucial role in disease prevention and control, serving as the foundation for subsequent preventive measures and research. The application of mNGS in livestock primarily focuses on the detection of prevalent viral diseases and the identification of novel viruses. Respiratory diseases, which significantly impact the livestock industry's economy, constitute a crucial category with multifactorial etiology. Bacteria and viruses are considered the primary pathogens responsible for respiratory diseases in livestock. Bouzalas et al. applied mNGS to study the medulla oblongata of cows suffering from non-septic encephalitis, and found a novel bovine astrovirus, BoAstV-CH13, with 92% genomic homology to BoAstV-NeuroSl. Subsequently, the authenticity of the mNGS assay was confirmed by the progress of RT-PCR and in situ hybridisation. Genetic evolutionary analysis of the viral genomes confirmed that BoAstV-CH13 and BoAstV-NeuroS1 were closely related to MiAstV-SMS, a mink tremor syndrome virus, and OvAstV-CH16, a sheep *Astrovirus*. It is noteworthy that BoAstV-CH13 and BoAstV-NeuroSl have not been detected to cause intestinal infections, unlike previous reports that bovine *Astroviruses* can cause intestinal diseases (9). Diarrhea is a prevalent disease in livestock and is caused by various factors, including bacteria, viruses, and parasites, as well as feeding and management practices. Viruses play a significant role in the etiology of diarrhea. Recently, mNGS has revealed novel viral strains potentially associated with diarrheal episodes in livestock samples. Keha-mo Abi and colleagues employed viral metagenomics techniques to identify a novel *Aichivirus* D strain in the fecal samples of Tibetan sheep suffering from diarrhea. A case-control study subsequently demonstrated that this virus was significantly associated with diarrhea in sheep and exhibited a high prevalence within the Qinghai-Tibet plateau (1). Qingxian Li et al. conducted a study to identify the causative agent of a diarrhea outbreak in a piglet herd in Luoyang City, Henan Province, China, in December 2022. Initially, four common viral agents known to cause diarrhea in piglets, including three *Coronavirus* and *Rotavirus* A (RVA), were tested; however, all tests yielded negative results. mNGS was subsequently employed to investigate potential pathogens present in other diarrhea samples, leading to the identification of a novel recombinant strain of *Rotavirus* B (RVB). These findings suggest

that the newly discovered recombinant RVB strain may be responsible for the occurrence of the diarrhea outbreak in the piglet herd (28). In 2018, Jinxin Xie and colleagues discovered a novel *Kirkovirus* in the virome of a deceased purebred mare from China that has specific infectivity toward intestinal cells and has the potential to spread within the equine population in northern Xinjiang (49).

In the past two decades, new infectious diseases have continuously emerged in avian species. The diversity of biological species and bird migration have provided favorable conditions for the spread of these diseases, resulting in significant economic losses to poultry farming. Identifying and characterizing novel avian viruses is crucial for the effective control of avian diseases. The complete genomes of four novel filamentous phages were identified in fecal samples from both wild and domestic birds by Jian Zeng and colleagues (53). Qifan Zhao et al. conducted a mNGS study on the fecal virome of black-necked cranes in Shigatse, Tibet. The viral community carried by these cranes predominantly consists of *Genomoviridae*, *Parvoviridae*, and *Picornaviridae*. Importantly, this is the initial detection of these viruses in black-necked crane fecal samples. This study offers valuable insights into the composition of the viral community within the gastrointestinal tract and holds significance for combating viral diseases affecting black-necked cranes (54).

The emergence of pathogens presents a significant threat to numerous wildlife species, particularly in rapidly evolving environments. Hence, the utilization of mNGS holds immense importance in uncovering previously unknown viruses harbored by wildlife, thereby facilitating a more comprehensive understanding of the diversity of wildlife viruses. Xinyuan Cui and colleagues identified a total of 27 species of mammalian viruses from 1,981 wild animals and 194 captive animals collected in southern China between 2015 and 2022. They successfully isolated eight viruses and comprehensively characterized their pathogenicity. Notably, bats present a high diversity of *Coronaviruses*, *Picornaviruses*, and *Astacoviruses*. Furthermore, this study revealed potential transmission pathways for *Picornaviruses* and respiratory viruses between bats and pangolins. Additionally, a novel branch of embecovirus and a new genus of *Arenaviruses* were discovered in Pikas. Furthermore, this study revealed the potential for cross-species transmission of RNA viruses (*Paramyxovirus* and *Astrovirus*) and DNA viruses (*Pseudorabies* virus, *Porcine Circovirus 2*, *Porcine Circovirus 3* and *Parvovirus*) between wildlife and domestic animals, thereby complicating the control of these diseases in both populations. This study offers a comprehensive perspective on the frequency of host-jumping events and an assessment of zoonotic risk (13). The pangolin, a wild mammal that faces significant threats, primarily due to illegal wildlife trade, was the subject of a study conducted by Wen Hua Gao et al., who utilized mNGS and reverse transcription polymerase chain reaction (RT-PCR) techniques to identify two previously unknown RNA viruses in deceased pangolins. Epidemiological investigations strongly suggest that these newly discovered viruses were likely introduced through illicit international trade involving pangolins. Consequently, these findings underscore the detrimental impact of illegal wildlife trafficking not only on pangolin populations but also on the potential dissemination of infectious disease agents (16). Kingsley et al. conducted a viral mNGS analysis of a fecal sample from a wild snow leopard, successfully identifying seven virus families and a novel *Bocaparvovirus* provisionally named *Panthera uncia bocaparvovirus* (PuBOV) (9). Rodents are geographically widespread wildlife that play crucial roles as reservoirs for numerous human and animal pathogens. Therefore, investigating the viral diversity harbored by rodents and identifying novel rodent-borne viruses is highly important in terms of preventing and controlling viral diseases (7). Tan et al. conducted a viral mNGS analysis of 314 wild rodents collected from northwest China and performed systematic virological identification of a novel *Wenzhou mammary tumor virus* (WENV) strain from a brown rat. The comprehensive genome sequencing and phylogenetic analysis results strongly support the classification of WENV as a newly discovered virus (42). Marie Horemans et al. conducted nanopore mNGS on 127 bat samples, 34 rodent samples, and 17 shrew samples collected in Belgium. Through their analysis, they identified six novel viruses belonging to the *Jeilongvirus* and *Henipavirus* genera. Additionally, they made a significant discovery by identifying a gene that encodes a protein with an unknown function within the genome of a newly discovered *Henipavirus* (23). The mouse family belongs to a diverse group of rodents that are widely distributed, encompass numerous species, and exhibit robust reproductive capabilities. They serve as significant reservoirs for various viruses and maintain close proximity to humans while harboring multiple pathogenic viruses capable of infecting both humans and animals (4, 10, 14). Using viral mNGS, Asif Mahmood et al. analyzed fecal samples obtained from 20 wild rats in Zhenjiang City, China. This study led to the identification of a novel *Cardiovirus* within one of the sampled wild rats and subsequent acquisition of its complete genome sequence. Epidemiological investigations revealed that this newly discovered *Cardiovirus* is limited to a single sample and does not exhibit widespread prevalence (29). Understanding the unclassified viruses carried by rodents in natural environments can offer scientific guidance for the prevention and control of emerging viral outbreaks.

Vector-borne viruses are transmitted by arthropod vectors and pose a significant threat to global public

health. Ticks, which are ubiquitous ectoparasites that feed on blood as their sole source of nutrition, can be found worldwide and are considered one of the most crucial disease vectors globally. They harbor various pathogens, including bacteria, and viruses. Tick-borne viruses present a substantial risk to both human and animal health. Research on tick-borne viruses plays a vital role in effectively identifying known and novel virus strains carried by ticks. Moreover, such research can identify the hosts, vectors, transmission ranges, systematic evolutionary history, and interactions of these viruses with other pathogens (45). To elucidate the viral characteristics associated with Australian ticks, particularly those potentially linked to mammalian infections, Erin Harvey et al. conducted mNGS in 146 ticks collected from two coastal sites in New South Wales, Australia. The findings revealed the presence of 19 novel RNA viruses belonging to diverse families. Notably, three of these viruses form clusters with known mammalian viruses, including a novel *E. coli* virus closely related to the human pathogen of *Colorado tick fever virus* (20). Mosquitoes are also vectors for transmitting vector-borne pathogens. Souand Mohamed Ali et al. employed mNGS to analyze the diversity of viruses carried by mosquitoes collected from various habitats in Cambodia. The samples revealed 26 virus lineages, including well-known vector-borne viruses, such as yellow fever viruses and *Orthobunyaviruses*, alongside a cluster of viruses that remain unclassified within taxonomy. This study demonstrated the vast diversity of viral communities while highlighting the presence of novel and unclassified viruses (31).

Microbial Community Structure Research. By conducting mNGS on samples collected from epidemic regions or outbreak sites of animal diseases, we can elucidate the population structure, transmission pathways, and evolutionary patterns of pathogenic microorganisms. These findings provide a scientific foundation for the diagnosis of epidemics. Zhou et al. initially documented the viral community associated with the Porcine Respiratory Disease Complex (PRDC) in Ganzi Tibetan Autonomous Prefecture, Sichuan Province. They demonstrated that coinfection with *Porcine Circovirus Type 2* (PCV2) and *Torque Teno Sus Virus Type 2* (TTSuV2) was the primary causative factor for PRDC in Tibetan pigs. Analysis of viral genomic sequences revealed that PCV2 isolates belonged to the PCV2d lineage, whereas the TTSuV isolates were classified into genotypes TTSuV2a and TTSuV2b. This study represents the first report on the viral community associated with PRDC infection in Tibetan pigs within this region, providing valuable insights for prevention and control strategies against respiratory diseases in these animals (55). Wang et al. identified 251 virus genomes associated with vertebrates from a sample of 844 bats and 250 rodents collected in Kenya and Uganda, revealing the extensive viral diversity, host-specific variability, and pronounced geographical specificity of viruses in East Africa (43). To investigate arbovirus epidemics, He et al. collected a total of 22,959 mosquitoes from Shaanxi, Gansu, and the Ningxia Hui Autonomous region in China between June and August 2019. They employed high-throughput sequencing and mNGS analysis to discern the viral groups present in these mosquitoes. The findings identified 116 virus species belonging to 31 virus families, indicating that both mosquito species and collection time influenced the diversity and abundance of viruses (21).

Drug resistance gene research. The emergence of antimicrobial resistance presents a significant challenge to the control of animal diseases. In recent decades, numerous novel and transferable antibiotic resistance genes (ARGs) have been widely disseminated on a global scale (44). A comprehensive understanding of antibiotic resistance mechanisms in both culturable and unculturable bacteria can offer valuable insights into eliminating resistance, thereby enhancing drug design efficacy. Moreover, antibiotic resistance genes often coexist with synthetic genes within the microbial genome, thus providing guidance for nearby DNA biosynthesis pathways (39). Horizontal gene transfer (HGT) serves as the primary mechanism facilitating the dissemination of antibiotic resistance in both natural and clinical settings (22, 27), accounting for 75% of the exchange of antibiotic resistance between environmental microorganisms and clinical pathogens (2, 41). In recent years, mNGSs has emerged as a valuable tool for investigating antibiotic resistance genes because of its unbiased and high-coverage characteristics. Gupta et al. provided an in-depth discussion of the fundamental workflow of mNGS research on antibiotic resistance genes, encompassing sample nucleic acid extraction, quality control and data processing, sequencing, alignment, annotation of antibiotic resistance genes, downstream data and omics analysis. Additionally, they compared the advantages and applications of various antibiotic resistance gene databases and analysis methods. This study offers a practical reference for conducting mNGS research on antibiotic resistance genes (26). Intensive pig farms are recognized as major sites for the dissemination of antibiotic resistance genes (ARGs). Phages, which serve as crucial mobile carriers of ARGs, are widely prevalent in animal intestines. Ji et al. conducted mNGS and analysis of viral DNA and total DNA in the ileum and cecum of healthy piglets and those with diarrhea. The findings revealed that phages residing in the blind intestine of piglets were the primary repository for ARGs and mobile genetic element (MGE) genes. Phage-associated MGEs play a significant role in maintaining and transferring ARGs. This study significantly enhances our understanding of the porcine intestinal microbiome while revealing the

intrinsic mechanisms underlying ARG maintenance and transmission within pig intestines, thereby offering valuable insights for preventing and controlling ARG pollution in the livestock industry (25).

Revealing the relationships among pathogens, hosts and the environment. Establishing connections between host and virus diversity across a broad geographical range is crucial. mNGS can contribute to understanding, tracking, and combating infections more efficiently (Fig. 2) (30). Analyzing virus hosts in different regions can offer a broader perspective on virus ecology. Moreover, the collection of samples from diverse climate zones and habitats, followed by a comparison of their viral composition, is pivotal for understanding the biogeography of related viruses. By comparing the diversity and prevalence of viruses (particularly *Arboviruses*) across various regions, potential hotspots for infectious disease emergence can be identified, thereby providing valuable guidance for disease surveillance (34). Pan et al. employed metagenomic transcriptomics to elucidate the virome of 81 species encompassing 2,438 mosquitoes across an expansive span of approximately 4,000 kilometers in China. This comprehensive investigation revealed a total of 393 mosquito-associated viruses, including seven arthropod-borne viruses. Furthermore, this study identified mosquito species, characterized their viral diversity, pinpointed geographic hotspots for *Arbovirus* emergence, and confirmed that the composition of individual mosquito viromes is intricately linked to host phylogeny. These findings shed light on extensive virus sharing among mosquito species or genera and significantly contribute to our understanding of the host specificity of *Arboviruses*. This study also revealed that a wide range of viruses are extensively distributed throughout China, which may indicate the capacity of mosquitoes for long-distance transmission of viruses. In summary, these findings significantly broaden our understanding of the diversity of mosquito-borne viruses, establishing a connection between viral variation within a single insect and viral diversity at the national scale. Thus, they provide valuable insights into the biogeography and diversity of arboviruses (38). Patricia Gil et al. compare taxonomic diversity between different spatial scales in the eukaryotic virome of the mosquito *Culex pipiens*. They collected large numbers of mosquitoes in five countries in Africa and Europe around the Mediterranean Sea and found a group of viruses in all countries. However, the prevalence of certain core viruses fluctuated between countries and years. This illustrates the fact that viromes vary according to geographic location and that viromes tend to cluster according to continent (17). Zhu et al. randomly collected 1,120 fecal samples from 58 farms in northeastern China and categorized them into 72 groups based on clinical symptoms, age, breed of cattle, sex, farming pattern, and geographic location. mNGS was employed to compare the pathogen composition among the different groups. This study revealed a significant correlation between the numbers of viruses and bacteria and host factors, such as clinical status, cattle type, and age, as well as environmental factors, including farming patterns and geographic location. By investigating microbial-host-environment disease ecology, this study revealed novel ecological risk factors associated with cattle diarrhea and offered a fresh perspective for its control (57). Michelle Wille et al. investigated the impact of host age on virus communities by employing mNGS to analyze oropharyngeal and cloacal swabs from Ruddy Turnstones across different age groups. They successfully identified a total of 14 potential bird-infecting viruses, including 11 novel types, such as *Reoviridae*, *Astroviridae*, and *Picornaviridae*. Interestingly, all 12 identified viruses were exclusively found in first-year juveniles, with only two being detected in adult birds. Moreover, the viral abundance and alpha diversity were slightly greater in juveniles than in adults. These findings highlight the importance of considering host age when monitoring new and emerging viruses alongside studying viral ecology (46).

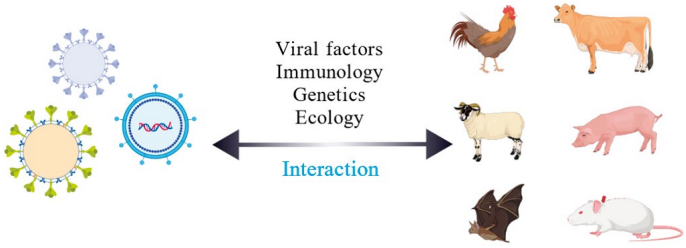

Fig. 2. mNGS help decipher ecological, immune, viral, and genetic factors that influence. Created with BioGDP.com

Limitations of metagenomic sequencing

As described in the previous sections, mNGS is a promising approach for pathogen detection that has demonstrated its effectiveness in numerous clinical trials, particularly in the identification of unknown viral strains. However, certain limitations remain associated with current mNGS methods.

The challenges associated with data processing, the high cost of sequencing, and the lengthy analysis cycle pose limitations on the application of mNGS in animal clinical disease diagnosis. Given the current state of knowledge, mNGS may not always facilitate prompt clinical decision-making due to significant variability in specialized knowledge, methods, and interpretations needed for sample preparation, sequencing, and bioinformatics analysis (6).

The results of mNGS are significantly influenced by external factors. Pathogen detection relies on the relative abundance of pathogen sequences within the entire sequencing library. Optimal mNGS performance is achieved when host DNA or pathogen sequences reach a specific proportion in the library (36). Although

mNGS is more sensitive than traditional methods are, mNGS is susceptible to false-positive results due to contamination from external environments, containers, and reagents. Therefore, the use of a negative control for mNGS is advisable (55).

The sensitivity of mNGS for virus detection is lower than those for bacteria and viruses because of the high diversity of viruses and their smaller genomes. Additionally, viral DNA in microbial community samples is often present in low proportions (18). To increase virus detection sensitivity, a research team in the United States has developed VirCapSeq-VERT, a vertebrate virome capture sequencing platform (10). Viruses also lack a marker gene analogous to the bacterial 16S gene, and the available reference sequences are extremely limited, rendering viruses unsuitable for amplicon sequencing-based analysis. Although mNGS can detect numerous viral sequences, the absence of a comprehensive virus metagenome database and the inability to match a substantial number of viral sequences with this database pose significant challenges in annotating the functional roles of unknown organisms in virus research (32). The pathogenicity of certain animal viruses can be detected solely via mNGS because they cannot be effectively isolated and identified. Consequently, definitively identifying the causative agent via Koch's postulates becomes unfeasible, which suggests a potential association between the pathogen and animal diseases (48).

The utilization of high-throughput sequencing technology generates a substantial volume of data, necessitating the comparison and analysis of detection results against established microbial genomic databases. However, the incompleteness of pathogen-specific genomic databases may result in the potential omission of certain rare pathogens (47). Different databases and interpreters can lead to a lack of uniform standards and consequently different detection results. To avoid these limitations, further optimization is needed in terms of sequencing throughput, accuracy, automation, sequencing cost and time, and the richness and ease of use of bioinformatics data analysis software (15).

With the changing global climate and ecological conditions, the gradual loss of biodiversity has led to a surge in animal diseases worldwide. Traditional detection technologies are no longer adequate for effective animal disease diagnosis, particularly when addressing unknown diseases. mNGS is characterized by high sensitivity, nontargeted analysis, and high-throughput capabilities, making it an invaluable adjunct to traditional detection technologies in animal disease control efforts. Although mNGS cannot entirely replace conventional pathogen detection methods, the results obtained through mNGS can provide valuable information and guidance for monitoring and controlling animal diseases. As science and technology continue to advance and improve, mNGS will undoubtedly find wider applications in future endeavors related to animal disease treatment and diagnosis.

References

1. *Abi K. M., Yu Z, Jing Z. Z., Tang C.*: Identification of a novel Aichivirus D in sheep. Infect. Genet. Evol. 2021, 91, 104810, doi: 10.1016/j.meegid.2021.104810.
2. *Aminov R. I.*: Horizontal gene exchange in environmental microbiota. Front. Microbiol. 2011, 2, 158, doi: 10.3389/fmicb.2011.00158.
3. *Amrane S., Raoult D., Lagier J. C.*: Metagenomics, culturomics, and the human gut microbiota. Expert. Rev. Anti. Infect. Ther. 2018, 16 (5), 373-375, doi: 10.1080/14787210.2018.1467268.
4. *Barbosa C. M., Soares R. M., Gregori F.*: Synanthropic rodents as virus reservoirs and transmitters. Rev. Soc. Bras. Med. Trop. 2020, 53, e20190486, doi: 10.1590/0037-8682-0486b-2019.
5. *Bass D., Stentiford G. D., Wang H. C., Koskella B., Tyler C. R.*: The pathobiome in animal and plant diseases. trends. Ecol. Evol. 2019, 34 (11), 996-1008, doi: 10.1016/j.tree.2019.07.012.
6. *Batool M., Galloway-Peña J.*: Clinical metagenomics-challenges and future prospects. Front. Microbiol. 2023, 14, 1186424, doi: 10.3389/fmicb.2023.1186424.
7. *Bordes F., Blasdell K., Morand S.*: Transmission ecology of rodent-borne diseases.: New frontiers. Integr. Zool. 2015, 10 (5), 424-435, doi: 10.1111/1749-4877.12149.
8. *Both G. W., Sleigh M., Cox N., Kendal A.*: Antigenic drift in influenza virus H3 hemagglutinin from 1968 to 1980.: multiple evolutionary pathways and sequential amino acid changes at key antigenic sites. J. Virol. 1983, 48, 52-60, doi: 10.1128/JVI.48.1.52-60.1983.
9. *Bouzalas I. G., Wüthrich D., Walland J., Drögemüller C., Zurbriggen A., Vandevelde M., Oevermann A., Bruggmann R., Seuberlich T.*: Neurotropic astrovirus in cattle with nonsuppurative encephalitis in Europe. J. Clin. Microbiol. 2014, 52 (9), 3318-3324, doi: 10.1128/JCM.01195-14.
10. *Briese T., Kapoor A., Mishra N., Jain K., Kumar A., Jabado O. J., Lipkin W. I.*: Virome capture sequencing enables sensitive viral diagnosis and comprehensive virome analysis. mBio. 2015, 6 (5), e01491-15, doi: 10.1128/mBio.01491-15.
11. *Burgin C. J., Colella J. P., Kahn P. L., Upham N. S.*: How many species of mammals are there? J. Mammal. 2018, 99, 1-14, doi: 10.1093/jmammal/gyx147.
12. *Chukwudozie K. I., Wang H., Wang X., Lu C., Xue J., Zhang W., Shan T.*: Viral metagenomic analysis reveals diverse viruses and a novel bocaparvovirus in the enteric virome of snow leopard (Panthera uncia). Heliyon 2024, 10 (8), e29799, doi: 10.1016/j.heliyon.2024.e29799.
13. *Cui X., Fan K., Liang X., Gong W., Chen W., He B., Chen X., Wang H., Wang X., Zhang P., Lu X., Chen R., Lin K., Liu J., Zhai J., Liu D. X., Shan F., Li Y., Chen R. A., Meng H., Li X., Mi S., Jiang J., Zhou N., Chen Z., Zou J. J., Ge D., Yang Q., He K., Chen T., Wu Y. J., Lu H., Irwin D. M., Shen X., Hu Y., Lu X., Ding C., Guan Y., Tu C., Shen Y.*: Virus diversity, wildlife-domestic animal circulation and potential zoonotic viruses of small mammals, pangolins and zoo animals. Nat. Commun. 2023, 14 (1), 2488, doi: 10.1038/s41467-023-38202-4.
14. *Dahmana H., Granjon L., Diagne C., Davoust B., Fenollar F., Mediannikov O.*: Rodents as Hosts of Pathogens and Related Zoonotic Disease Risk. Pathogens. 2020, 9, 202, doi: 10.3390/pathogens9030202.
15. *Diao Z., Han D., Zhang R., Li J.*: Metagenomics next-generation sequencing tests take the stage in the diagnosis of lower respiratory tract infections. J. Adv. Res. 2021, 38, 201-212, doi: 10.1016/j.jare.2021.09.012.
16. *Gao W. H., Lin X. D., Chen Y. M., Xie C. G., Tan Z. Z., Zhou J. J., Chen S., Holmes E. C., Zhang Y. Z.*: Newly identified viral genomes in pangolins with fatal disease. Virus. Evol. 2020, 6 (1), veaa020, doi: 10.1093/ve/veaa020.
17. *Gil P., Exbrayat A., Loire E., Rakotoarivony I., Charriat F., Morel C., Baldet T., Boisseau M., Marie A., Frances B., L'Ambert G., Bessat M., Otify Y., Goffredo M., Mancini G., Busquets N., Birnberg L., Talavera S., Aranda C., Ayari E., Mejri S., Sghaier S., Bennouna A., El Rhaffouli H., Balenghien T., Chlyeh G., Fassi Fihri O., Reveillaud J., Simonin Y., Eloit M., Gutierrez S.*: Spatial scale influences the distribution of viral diversity in the eukaryotic virome of the mosquito Culex pipiens. Virus Evol. 2023 Aug 24; 9 (2):vead054, doi: 10.1093/ve/vead054.
18. *Gu W., Miller S., Chiu C. Y.*: Clinical metagenomic next-generation sequencing for pathogen detection. Annu. Rev. Pathol. 2019, 14, 319-338, doi: 10.1146/annurev-pathmechdis-012418-012751.
19. *Hardinge P., Murray J. A. H.*: Reduced false positives and improved reporting of loop-mediated isothermal amplification using quenched fluorescent primers. Sci. Rep. 2019, 9 (1), 7400, doi: 10.1038/s41598-019-43817-z.
20. *Harvey E., Rose K., Eden J. S., Lo N., Abeyasuriya T., Shi M., Doggett S. L., Holmes E. C.*: Extensive diversity of RNA viruses in Australian ticks. J. Virol. 2019, 93 (3), e01358-18, doi: 10.1128/JVI.01358-18.

21. *He X., Yin Q., Zhou L., Meng L., Hu W., Li F., Li Y., Han K., Zhang S., Fu S., Zhang X., Wang J., Xu S., Zhang Y., He Y., Dong M., Shen X., Zhang Z., Nie K., Liang G., Ma X., Wang H.*: Metagenomic sequencing reveals viral abundance and diversity in mosquitoes from the Shaanxi-Gansu-Ningxia region, China. PLoS. Negl. Trop. Dis. 2021, 15 (4), e0009381, doi: 10.1371/journal.pntd.0009381.
22. *Hooton S., Millard A., Baker M., Stekel D., Hobman J.*: DNA Traffic in the environment and antimicrobial resistance. Springer. Singapore 2019, 245-271, doi: 10.1007/978-981-13-3411-5_10.
23. *Horemans M., Van Bets J., Joly Maes T., Maes P., Vanmechelen B.*: Discovery and genome characterization of six new orthoparamyxoviruses in small Belgian mammals. Virus Evol. 2023, 11, 9 (2): vead065, doi: 10.1093/ve/vead065.
24. *Hugenholtz P., Tyson G. W.*: Microbiology: metagenomics. Nature 2008, 455 (7212), 481-483, doi: 10.1038/455481a.
25. *Ji Y., Xi H., Zhao Z., Jiang Q., Chen C., Wang X., Li F., Li N., Sun C., Feng X., Lei L., Han W., Gu J.*: Metagenomics analysis reveals potential pathways and drivers of piglet gut phage-mediated transfer of ARGs. Sci. Total. Environ. 2023, 859 (Pt 2), 160304, doi: 10.1016/j.scitotenv.2022.160304.
26. *Lal Gupta C., Kumar T. R., Cytryn E.*: Platforms for elucidating antibiotic resistance in single genomes and complex metagenomes. Environ. Int. 2020, 138, 105667, doi: 10.1016/j.envint.2020.105667.
27. *Lerminiaux N. A., Cameron A. D. S.*: Horizontal transfer of antibiotic resistance genes in clinical environments. Can. J. Microbiol. 2019, 65 (1), 34-44, doi: 10.1139/cjm-2018-0275.
28. *Li Q., Wang Z., Jiang J., He B., He S., Tu C., Guo Y., Gong W. L.*: Outbreak of piglet diarrhea associated with a new reassortant porcine rotavirus B. Vet. Microbiol. 2024, 288, 109947, doi: 10.1016/j.vetmic.2023.109947.
29. *Mahmood A., Shama S., Ni H., Wang H., Ling Y., Xu H., Yang S., Naseer Q. A., Zhang W.*: Viral metagenomics revealed a novel cardiovirus in feces of wild rats. Intervirology. 2019, 62 (1), 45-50, doi: 10.1159/000500555.
30. *Medeiros R. M. de, Valverde-Villegas J. M., Ellwanger J. H.*: Editorial.: Host-virus interaction at the omics and ecology levels. Front. Immunol. 2023, 14, 1209532, doi: 10.3389/fimmu.2023.1209532.
31. *Mohamed A. S., Rakotonirina A., Heng K., Jacquemet E., Volant S., Temmam S., Boyer S., Eloit M.*: Longitudinal study of viral diversity associated with mosquito species circulating in Cambodia. Viruses 2023, 15 (9), 1831, doi: 10.3390/v15091831.
32. *Moore-Connors J. M., Dunn K. A., Bielawski J. P., Van L. J.*: Novel strategies for applied metagenomics. Inflamm. Bowel. Dis. 2016, 22 (3), 709-718, doi: 10.1097/MIB.0000000000000717.
33. *Mullis K., Faloona F., Scharf S., Saiki R., Horn G., Erlich H.*: Specific enzymatic amplification of DNA in vitro.: the polymerase chain reaction. Cold. Spring. Harb. Symp. Quant. Biol. 1986, 51 (Pt 10), 263-273, doi: 10.1101/sqb.1986.051.01.032.
34. *Murray K. A., Preston N., Allen T., Zambrana-Torrelio C., Hosseini P. R., Daszak P.*: Global biogeography of human infectious diseases. Proc. Natl. Acad. Sci. USA 2015, 112 (41), 12746-12751, doi: 10.1073/pnas.1507442112.
35. *Nadon C., Van Walle I., Gerner-Smidt P., Campos J., Chinen I., Concepcion-Acevedo J., Gilpin B., Smith A. M., Man Kam K., Perez E., Trees E., Kubota K., Takkinen J., Nielsen E. M., Carleton H., FWD-NEXT Expert Panel.*: PulseNet international.: vision for the implementation of whole genome sequencing (WGS) for global food-borne disease surveillance. Euro. Surveill. 2017, 22 (23), 30544, doi: 10.2807/1560-7917.ES.2017.22.23.30544.
36. *Olausson J., Brunet S., Vracar D., Tian Y., Abrahamsson S., Meghadri S., Sikora H. P., Lind Karlberg M., Jakobsson H. E., Tang K. W.*: Optimization of cerebrospinal fluid microbial DNA metagenomic sequencing diagnostics. Sci. Rep. 2022, 12 (1), 3378, doi: 10.1038/s41598-022-07260-x.
37. *Pace N. R., Stahl D. A., Lane D. J., Olsen G. J.*: The analysis of natural microbial populations by ribosomal RNA sequences. Advances in Microbial Ecology 1986, 9, 1-55, doi: 10.1007/978-1-4757-0611-6_1.
38. *Pan Y. F., Zhao H., Gou Q. Y., Shi P. B., Tian J. H., Feng Y., Li K., Yang W. H., Wu D., Tang G., Zhang B., Ren Z., Peng S., Luo G. Y., Le S. J., Xin G. Y., Wang J., Hou X., Peng M. W., Kong J. B., Chen X. X., Yang C. H., Mei S. Q., Liao Y. Q., Cheng J. X., Wang J., Chaolemen., Wu Y. H., Wang J. B., An T., Huang X., Eden J. S., Li J., Guo D., Liang G., Jin X., Holmes E. C., Li B., Wang D., Li J., Wu W. C., Shi M.*: Metagenomic analysis of individual mosquito viromes reveals the geographical patterns and drivers of viral diversity. Nat. Ecol. Evol. 2024, 8 (5), 947-959, doi: 10.1038/s41559-024-02365-0.
39. *Sabree Z. L., Rondon M. R., Handelsman J.*: Metagenomics. Encyclopedia Microbiol. 2009, 622-632, doi: 10.1016/B978-012373944-5.00034-1.
40. *Schmidt T. M., DeLong E. F., Pace N. R.*: Analysis of a marine picoplankton community by 16S rRNA gene cloning and sequencing. J. Bacteriol. 1991, 173 (14), 4371-4378, doi: 10.1128/jb.173.14.4371-4378.
41. *Soucy S. M., Huang J., Gogarten J. P.*: Horizontal gene transfer.: building the web of life. Nat. Rev. Genet. 2015, 16 (8), 472-482, doi: 10.1038/nrg3962.
42. *Tan Z., Yu H., Xu L., Zhao Z., Zhang P., Qu Y., He B., Tu Cl.*: Virome profiling of rodents in Xinjiang uygur autonomous region, China: Isolation and characterization of a new strain of Wenzhou virus. Virology 2019, 529, 122-134, doi: 10.1016/j.virol.2019.01.010.
43. *Wang D., Yang X., Ren Z., Hu B., Zhao H., Yang K., Shi P., Zhang Z., Feng Q., Nawenja C. V., Obanda V., Robert K., Nalikka B., Waruhiu C. N., Ochola G. O., Onyuok S. O., Ochieng H., Li B., Zhu Y., Si H., Yin J., Kristiansen K., Jin X., Xu X., Xiao M., Agwanda B., Ommeh S., Li J., Shi Z. L.*: Substantial viral diversity in bats and rodents from East Africa: insights into evolution, recombination, and cocirculation. Microbiome 2024, 12 (1), 72, doi: 10.1186/s40168-024-01782-4.
44. *Wee B. A., Muloi D. M., van Bunnik B. A. D.*: Quantifying the transmission of antimicrobial resistance at the human and livestock interface with genomics. Clin. Microbiol. Infect. 2020, 26 (12), 1612-1616, doi: 10.1016/j.cmi.2020.09.019.
45. *Wille M., Harvey E., Shi M., Gonzalez-Acuña D., Holmes E. C., Hurt A. C.*: Sustained RNA virome diversity in Antarctic penguins and their ticks. ISME. J. 2020, 14 (7), 1768-1782, doi: 10.1038/s41396-020-0643-1.
46. *Wille M., Shi M., Hurt A. C., Klaassen M., Holmes E. C.*: RNA virome abundance and diversity is associated with host age in a bird species. Virology 2021, 561, 98-106, doi: 10.1016/j.virol.2021.06.007.
47. *Wilson M. R., Sample H. A., Zorn K. C., Arevalo S., Yu G., Neuhaus J., Federman S., Stryke D., Briggs B., Langelier C., Berger A., Douglas V., Josephson S. A., Chow F. C., Fulton B. D., DeRisi J. L., Gelfand J. M., Naccache S. N., Bender J., Dien Bard J., Murkey J., Carlson M., Vespa P. M., Vijayan T., Allyn P. R., Campeau S., Humphries R. M., Klausner J. D., Ganzon C. D., Memar F., Ocampo N. A., Zimmermann L. L., Cohen S. H., Polage C. R., DeBiasi R. L., Haller B., Dallas R., Maron G., Hayden R., Messacar K., Dominguez S. R., Miller S., Chiu C. Y.*: Clinical metagenomic sequencing for diagnosis of meningitis and encephalitis. N. Engl. J. Med. 2019, 380 (24), 2327-2340, doi: 10.1056/NEJMoa1803396.
48. *Xie G., Yu J., Duan Z.*: New strategy for virus discovery: viruses identified in human feces in the last decade. Sci. China Life Sci. 201, 56 (8), 688-696, doi: 10.1007/s11427-013-4516-y.
49. *Xie J., Tong P., Zhang A., Yan Y., Zhang L., Song X., Chen J., Zhai S., Sha Y. N., Wang D., Su Z., Kuang L.*: First detection and genetic characterization of a novel kirkovirus from a dead thoroughbred mare in northern Xinjiang, China, in 2018. Arch. Virol. 2020, 165 (2), 403-406, doi: 10.1007/s00705-019-04487-4.
50. *Yang D. M., Chang T. J., Wang M. L., Tsai P. H., Lin T. H., Wang C. T., Liang K. H.*: Hunting severe acute respiratory syndrome coronavirus 2 (2019 novel coronavirus): From laboratory testing back to basic research. J. Chin. Med. Assoc. 2020, 83 (6), 524-526, doi: 10.1097/JCMA.0000000000000332.
51. *Yang S., Rothman R. E.*: PCR-based diagnostics for infectious diseases: uses, limitations, and future applications in acute-care settings. Lancet Infect Dis. 2004 Jun; 4 (6), 337-348, doi: 10.1016/S1473-3099(04)01044-8.
52. *Yang X., Yang J., Wang H.*: Advances in diagnosis technologies of viral animal epidemic diseases. China Anim. Husb. Vet. Med. 2022, 49 (01), 294-306, doi: 10.16431/j.cnki.1671-7236.2022.01.032.
53. *Zeng J., Wang Y., Zhang J., Yang S., Zhang W.*: Multiple novel filamentous phages detected in the cloacal swab samples of birds using viral metagenomics approach. Virol. J. 2021, 18 (1), 240, doi: 10.1186/s12985-021-01710-0.
54. *Zhao Q., Zhao R., Sun Y., Ji L., Xi Y., Wang X., Shen Q., Ji L., Wang Y., You Z., Yang S., Zhang W.*: Identification of multiple novel viruses in fecal samples of black-necked cranes using viral metagenomic methods. Viruses 2023, 15 (10), 2068, doi: 10.3390/v15102068.
55. *Zhao Y., Zhang W., Zhang X.*: Application of metagenomic next-generation sequencing in the diagnosis of infectious diseases. Front. Cell Infect. Microbiol. 2024, 14, 1458316, doi: 10.3389/fcimb.2024.1458316.
56. *Zhou L., Zhou H., Fan Y., Wang J., Zhang R., Guo Z., Li Y., Kang R., Zhang Z., Yang D., Liu J.*: Metagenomics to identify viral communities associated with porcine respiratory disease complex in Tibetan pigs in the Tibetan plateau, China. Pathogens 2024, 13 (5), 404, doi: 10.3390/pathogens13050404.
57. *Zhu Q., Qi S., Guo D., Li C., Su M., Wang J., Li Z., Yang D., Sun H., Wang X., Wang M., Wu H., Yu S., Bai W., Zhang Y., Yang X., Jiang L., Liu J., Zhao Y., Xing X., Shi D., Feng L., Sun D.*: A survey of fecal virome and bacterial community of the diarrhea-affected cattle in northeast China reveals novel disease-associated ecological risk factors. mSystems 2024, 9 (1), e0084223, doi: 10.1128/msystems.00842-23.

Corresponding authors:

Ai Jun, researcher, PhD, Technology Center of Kunming Customs, Xishan district, Kunming City, Yunnan Province, People's Republic of China; e-mail: 13769143048@163.com

Xin Jige, professor, PhD, Yunnan Agricultural University, Panlong district, Kunming City, Yunnan Province, People's Republic of China; e-mail: 2007032@ynau.edu.cn.