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CASE REPORT

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Metagenomic next-generation sequencing for etiological diagnosis of an unexpected rabies case with unclear exposure history

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Abstract

Background Rabies is an acute and lethal zoonotic disease caused by the rabies virus (RABV). After onset, there are no effective drugs or treatment methods.

Case presentation A 49-year-old female from Hefei, Anhui Province, China, presented to a local hospital with fever, pruritus, chest distress, and shortness of breath. During the consultation, the patient exhibited agitation and was later admitted to the intensive care unit (ICU) in the local hospital for endotracheal intubation and mechanical ventilation due to worsened agitation and dyspnea. Cerebrospinal fluid (CSF) and blood samples were collected and pathogenic microorganism identification was performed by culture and mNGS. However, all results were negative. In addition, the patient did not display typical rabies-specific symptoms such as aerophobia, hydrophobia or photophobia from onset to admission. Subsequently, saliva samples were collected for mNGS detection following consultation with experts at our hospital. Nucleic acid sequences uniquely aligned to the rabies virus (RABV) were identified in these samples. The result was further confirmed by local Center for Disease Control and Prevention (CDC) through RT-qPCR which detected part of the N gene of RABV in the saliva sample. The patient was then transferred to the ICU for isolation. Unfortunately, the patient died on the 10th day of admission due to multiple organ failure. The detection of human rabies virus IgG antibodies reported positive during the advanced stage of the disease during the hospitalization. We consistently verified with the patient's family member that there was no clear history of animal bites and no history of RABV vaccination. Furthermore, we performed phylogenetic analysis of partial L and G gene sequences of RABV obtained by mNGS (designated HFG23-L and HFG23-G, respectively), the results showed that both HFG23-L and HFG23-G belonged to the China I lineage, and shared 99.7% similarity with the Fengtai strain isolated from dogs in Beijing.

Conclusions The identification of unique RABV sequence through mNGS in the patient's saliva sample suggested that mNGS could serve as a valuable screening tool for the etiological diagnosis of rabies, especially when timely laboratory testing was unavailable or when patients lacked non-specific prodromal symptom and clear exposure history.

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Keywords Rabies, Rabies virus, Saliva, Metagenomic next-generation sequencing, Phylogenetic analysis, Unclear exposure history

Background

Human rabies is a deadly disease caused by the virus *Rabies lyssavirus* (RABV), which is a negative strand (antisense) RNA virus belonging to the family *Rhabdoviridae* [1]. In China, dog-associated RABV is the main cause of human rabies [2]. RABV is present in the saliva of wild and domestic infected-dogs and in the tissues at the injury site [1]. RABV infection in humans is characterized by a progression through four clinical stages: incubation, prodrome, acute neurological signs of encephalomyelitis, and ultimately death [3]. The primary clinical symptoms of rabies include aerophobia, hydrophobia, photophobia, dysphagia, mania, acute fatal encephalomyelitis and paralysis. Once rabies symptoms occur, the mortality rate of human is 100% [4]. The disease may present as furious or paralytic rabies. Furious rabies usually has obvious clinical symptoms, and patients die after a few days due to cardiopulmonary arrest. Paralytic rabies accounts for about 20% of all human cases. The paralytic form of rabies causes progressive muscle paralysis starting at the site of the bite or scratch and ending in death [5]. However, in some cases these typical clinical symptoms are not always present, which can make clinical diagnosis more difficult.

A total of 3,302 rabies cases were reported from 2015 to 2021 in China [6]. Anhui province is divided into medium epidemic regions of rabies. In 2017, there were 28,243 persons obtaining postexposure prophylaxis treatment from clinics, and 39 rabies cases were reported in Anhui province. Furthermore, 12 rabies cases were reported in Anhui province in 2021 [6]. Though the number of rabies cases is declining in China from 2015, RABV is still a fatal factor for human once infected.

To surveil and control rabies, early and accurate diagnosis is essential for clinicians. Clinical diagnosis and lab testing are always used to diagnose rabies [7]. However, it is difficult for clinicians to diagnose rabies without typical clinical symptoms (including hydrophobia and aerophobia) and a clear exposure history (wild/domestic animal bites or scratch) [8]. Lab testing methods are not applied for rabies detection in the absence of a clinical diagnosis, resulting in missing the detection of RABV [9].

Metagenomic next-generation sequencing (mNGS) is able to theoretically detect all pathogens in clinical samples within 24 h [10]. mNGS is still capable of identifying infectious agents when lab testing results are negative. In a study of Chinese population, from 2005 to 2019, the Chinese CDC collected 271 samples (including saliva, CSF, serum, and urine, as well as brain tissue, neck skin tissue, and cornea) from 164 suspected rabies cases from local hospitals. All of the samples were tested by RT-PCR, which showed that saliva samples had the highest positivity rate (32%), compared with CSF (around 21%) and other fluids [11]. Therefore, for RABV detection, saliva samples are preferred, followed by CSF samples and blood samples.

Case presentation

A 49-year-old woman presented with unexplained fever and pruritus, which did not improve after self-administration of antipyretic medication. Because influenza was suspected, the patient was treated with antiviral treatment at a local clinic the following day, but the patient's symptoms were not relieved. Subsequently, the patient was admitted to a local hospital due to chest distress and shortness of breath. Based on blood test results, potassium supplementation was initiated for the treatment of hypokalemia. Throughout the course of treatment, the patient exhibited agitation. Consultations with neurology and psychiatry specialists failed to alleviate or ameliorate the patient's manic symptoms which continued to worsen. Due to concurrent complaints of chest congestion, dyspnea and respiratory distress, the patient was transferred to the intensive care unit (ICU) for endotracheal intubation and mechanical ventilation. Five days later, the patient underwent bedside temporary cardiac pacemaker treatment due to an unstable heart rate. During hospitalization, based on the aforementioned symptoms, the physician suspected infection. CSF and blood samples were collected from the patient for culture identification. Additionally, CSF was subjected to metagenomic next-generation sequencing (mNGS). However, both culture and sequencing results were negative. Due to lack of diagnosis, several specialists from our hospital were invited for consultation. According to the patient's clinical symptoms and manifestations, doctors recommended further investigation into potential pathogenic agents. Therefore, saliva samples from the patient were sent to our hospital's laboratory for mNGS detection on 6 December 2023. On 7 December, mNGS results showed that the patient was infected by RABV, with 1566 unique viral reads and 89.06% coverage rate of the RABV genome (Fig. 1A).

The patient was admitted to the ICU of the previous hospital due to chest tightness and shortness of breath, and was transferred to ICU of our hospital for isolation treatment on 7 December 2023. The patient presented with unconsciousness and exhibited a temperature of 37.3 °C, pulse rate of 102 beats per minute, respiratory rate of 16 breaths per minute, and blood pressure

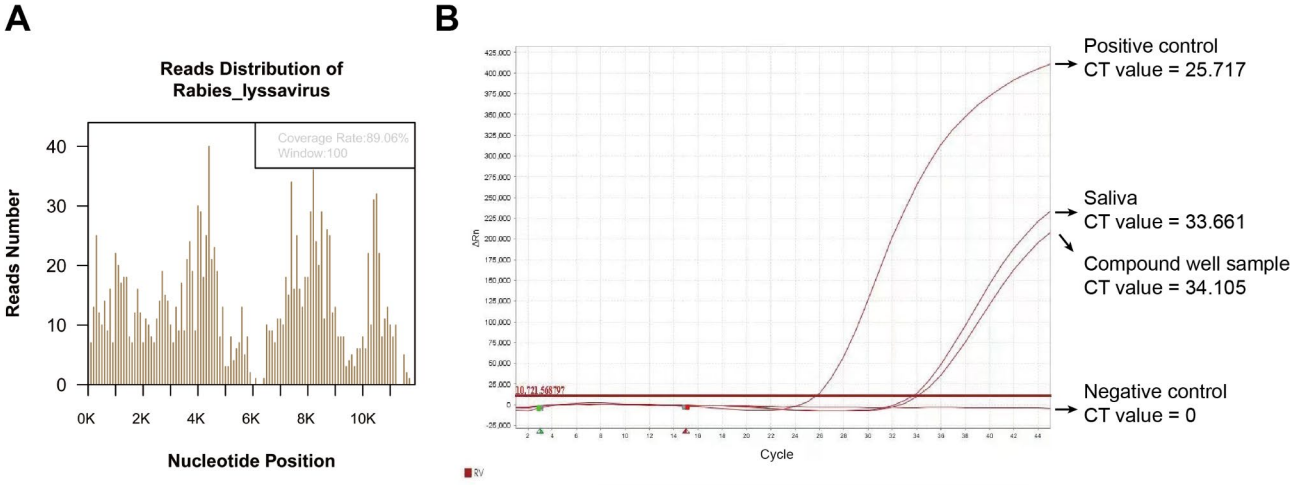

Fig. 1 The metagenomic next-generation sequencing and polymerase chain reaction (PCR) results of RABV. Distribution map of rabies virus genome reads detected in the laboratory (**A**), Detection of rabies virus amplification curve in specimens by RT-qPCR (**B**)

Table 1 The primers of RT-qPCR

Name	Sequence	Position in *N* gene	Size
RV-F1	AGTCTCCYTAYTCATCRAATGC	889–993	105
RV-R1	GCAATAACTGTYGCATTRAGAG		
RV-R2	GCAATRACTGTCGCATTAAGAG		
RV-PF (FAM, BQ1)	CATATAGCATCCRACAAAGTGAATGAG		

of 92/57 mmHg. Unequal pupil size (anisocoria) was observed, along with sensitivity to light and blood stains in the oral cavity. The neck displayed supple and lack of resistance. Intubation was performed, and ventilation was supported by a ventilator, accompanied by coarse breath sounds bilaterally in both lungs. No evident sputum rales were present, and bilateral Babinski sign as well as meningeal irritation sign were negative. The abdomen appeared flat, soft, and non-tender without any signs of edema in both lower limbs or jaundice on the skin or mucosa throughout the body. Additionally, no deformity of the head was noted. The patient did not exhibit any rabies-specific symptoms, such as aerophobia, hydrophobia, or photophobia, since the onset of fever and pruritus. After the detection of the RABV sequence through mNGS, the same saliva sample was sent to the local CDC on 7 December for thorough examination, and presence of the RABV was confirmed by RT-qPCR after five days (Fig. 1B; Table 1). The patient had been in a comatose state, and the progression of the disease was accompanied by elevated white blood cell count, increased levels of C-reactive protein, serum amyloid A, liver and kidney function indicators, electrolyte imbalance, continuous decline in red blood cells count and hemoglobin levels, intermittent temperature elevation, unstable pulse (Fig. 2; Table 2), sialorrhea, and quadriplegia. During the initial four days of hospitalization, the patient had a Glasgow Coma Scale score of 4T. On the fifth day of admission, there was a progressive decrease in blood pressure observed. At the same time, the detection of human rabies virus IgG antibodies (The Human Rabies Virus IgG Rapid Test Kit (Colloidal Gold), Beijing Beier Bioengineering Co., Ltd, Beijing, China) was conducted which yielded a positive result. The patient had not received prior rabies vaccination. Based on the presence of pruritus, accompanied by dysphoria, respiratory failure, and arrhythmia, the diagnosis of rabies was confirmed for this patient. The patient was managed with comprehensive supportive care in ICU, including respiratory support, fluid resuscitation, antimicrobial therapy, nutrition supplementation, and other appropriate symptomatic interventions. However, the therapeutic response was not prominently observed. Unfortunately, the patient died to multiple organ failure ten days after admission.

Before the patient's saliva mNGS results indicated a RABV infection, the patient had been in a comatose state and unable to communicate directly or effectively. Therefore, we corroborated the history of animal bites with the patient's daughter, and confirmed that incident of small animal bites, particularly from dogs, did not happened to the patient. To uncover possible infectious source of RABV in this case, phylogenetic analysis was implemented. Partial sequence fragment of L (1963 bp) or G (1475 bp) gene was obtained (namely HFG23-L and HFG23-G, respectively), and grouped into China I lineage of rabies virus (Figs. 3 and 4). HFG23-L and HFG23-G both showed about 99.7% nucleotide similarity to the corresponding sequences of strain Fengtai RABV (KC660078). Fengtai strain was isolated from a dog in Beijing, China. The results indicated that RABV in this study, infecting 49-year-old female, may have originated from a rabid dog.

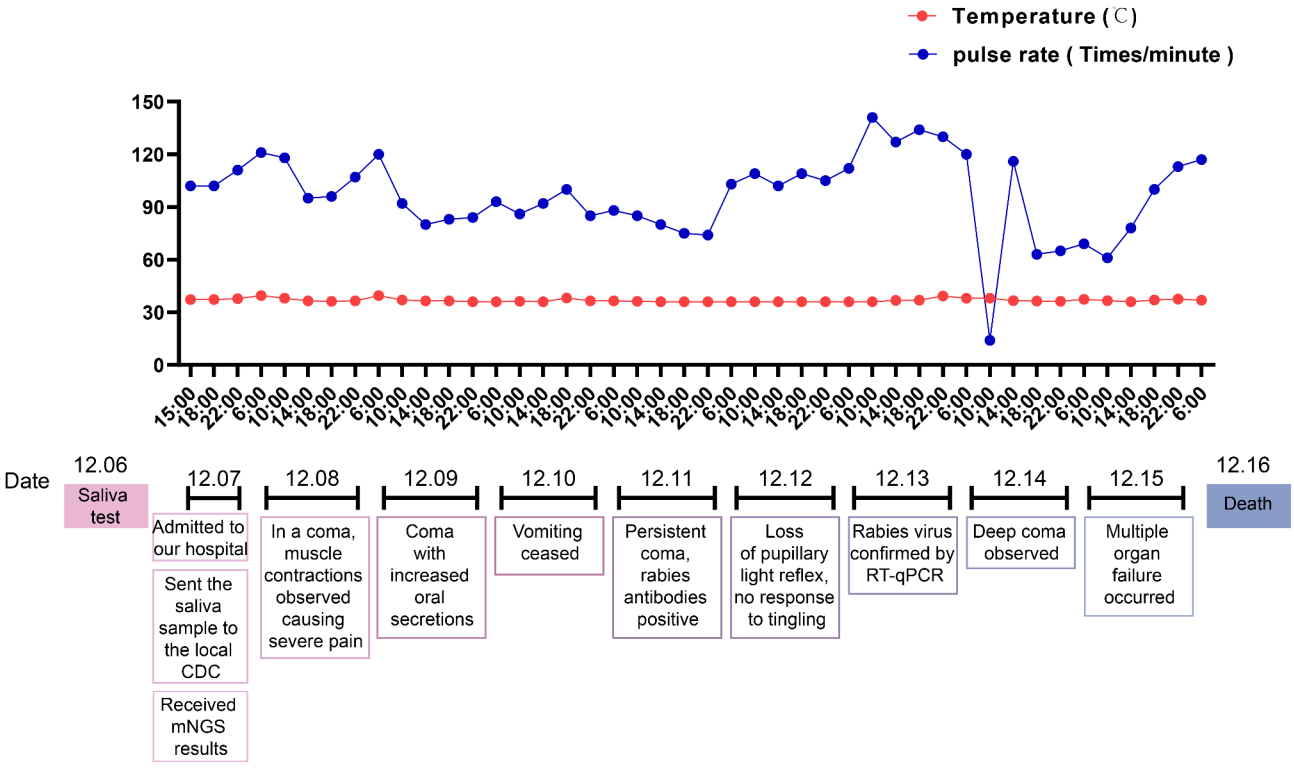

Fig. 2 Clinical course of the patient during hospitalization

Table 2 Partial laboratory testing results of patients in our hospital

Laboratory Test	2023.12.08	12.09	12.10	12.11	12.14	12.15	12.16
White blood cell (×109/L)	10.89	10.65			22.74	6.58	
C-reactive protein(mg/L)	23.89	14.61			124.21	210.28	
Red blood cell(×109/L)	3.78	3.32			3.32	2.62	
Hemoglobin(g/L)	79	70			72	57	
Prothrombin(s)	14				12.1		
D-Dimer(μg/ml)	4.94				2.06		
Alanine aminotransferase(U/L)	907	572	356		223		100
Aspartate aminotransferase(U/L)	684	172	60		173		101
Creatinine(μmol/L)	74	61	37		283	352	250
Glucose(mmol/L)	7.35	10.11			4.43		
K+(mmol/L)	4.72	4.65	4.6	4.49	7.85	6.45	3.62
Serum amyloid A(mg/L)	255	246.6			253.4		
Procalcitonin(ng/ml)		0.17			38.16		
creatine kinase-MB(ng/ml)	7	5				18	
lactic dehydrogenase(U/L)	787	348		209		266	
Myoglobin(ng/ml)	82	80		62		1203	
B-type natriuretic peptide(pg/ml)		3127	2330		> 35,000	> 35,000	
Sputum culture				negative			negative

Discussion

Timely diagnosis of rabies is indispensable for public health administration to control and surveil rabies [7]. In this study, the patient only felt itching and irritable mood in the early period, and then became comatose in a few days. The patient did not exhibit any rabies-specific symptoms of hydrophobia or aerophobia during the hospital visit. In addition, culture results of the patient's CSF and blood samples, as well as mNGS results of CSF, were negative, confusing clinicians to get an accurate diagnosis. For previously reported rabies cases, animal contact details were clear, and therefore, clinicians were able to get hints for rabies diagnosis on time [12–14]. Nevertheless, animal contact history was denied by the

Fig. 3 Phylogenetic analysis of G genes. ML (maximum-likelihood) tree of 107 partial G gene sequences. All sequences are truncated to 1317 bp after alignment. The fragment of HFG23-G gene (1317 bp) is located at positions 3575–4891 in reference genome (NC_001542.1) of rabies virus. China I-VI lineages are labeled with Roman numerals (I-VI), and HFG23-G gene is marked with red frame

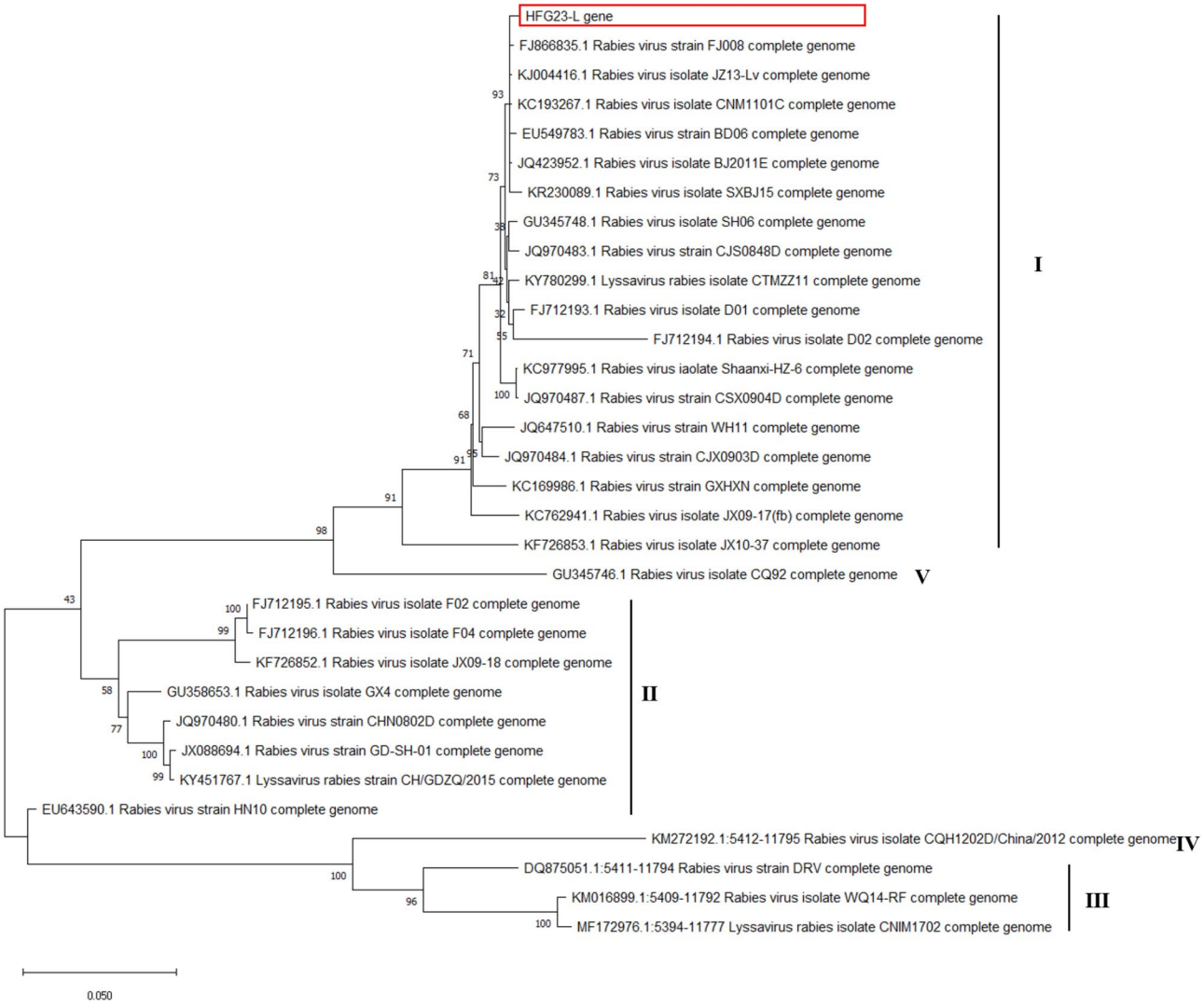

Fig. 4 Phylogenetic analysis of L genes. ML (maximum-likelihood) tree of 32 partial L gene sequences, and the length of all sequences is 1963 bp. The fragment of HFG23-L gene (1963 bp) is located at positions 7115–9077 in reference genome (NC_001542.1) of rabies virus. China I-V lineages are labeled with Roman numerals (I-V), and HFG23-L gene is marked with red frame

patient's daughter. Therefore, the patient was a great challenge for clinicians to make rabies diagnosis due to her quickly worsening situations and unclear causative reason. However, upon admission of the patient to our hospital's ICU for isolation treatment, RABV was identified in saliva samples from patient by mNGS. Previous research showed that positive rate of rabies in saliva samples (about 32%) is higher than that of CSF and blood samples [11]. It is recommended that saliva, rather than CSF or blood (serum), is considered as the primary choice for RABV detection in the stage of rabies development, particularly when prodromal symptoms are atypical, and exposure history is uncertain. mNGS may be a useful tool for clinical diagnosis of similar cases in the future [15–18]. In addition, more experience should be accumulated for accurate detection of rabies associated with non-specific clinical symptoms.

RABV sequences were assembled and analyzed by maximum-likelihood (ML) method. Phylogenetic tree of partial sequence of L and G gene showed that the virus belonged to China I lineage of RABV. China I lineage is a dog-associated prevalent lineage in China [19–20]. The homology of L and G gene partial sequences were about 99.7% with strain Fengtai rabies virus. G gene of rabies virus is conserved, and equivalent with N gene at phylogenetic position [21]. The similarity of G gene to virus strains isolated in Anhui was about 97%, indicating the virus was phylogenetically closer to Fengtai strain. It means that the virus may not belong to local rabies virus strains. For this patient, we did not obtain the complete virus genome, because additional samples could not be

collected after the patient's death. All the results demonstrated that the virus may have originated from a dog in this study. Although there was no certain dog bite or scratch history for the patient, current evidence inferred that the patient may have had contact with a dog.

The entire process of mNGS detection takes about 20–21 h and the RT-qPCR takes about 3–4 h. Theoretically, RT-qPCR takes shorter detection time than mNGS. However, for infections caused by unknown pathogens, the use of RT-qPCR may result in false negatives due to the pathogen not being within the pre-set target or the primers being unable to capture the target pathogen. For example, in the case of this report, it takes only 20–21 h for mNGS detection to be reported as RABV from collection to result report. The same saliva sample was subsequently sent to the CDC for reconfirmation by RT-qPCR. Although the RT-qPCR was less time-consuming, when the first and second reagents were negative, it took a week to find the third reagent to report the results (Supplementary Table S1). Therefore, mNGS has a greater advantage in the time of unknown pathogen detection.

The differences in RT-qPCR results may be due to the different detection targets of these commercial kits, and the L and G genes are not often used by commercial kits. In our case, partial sequences of L and G genes were obtained from saliva samples, although two small N gene fragments (< 500 bp) were assembled according to the results of mNGS, but only one N gene fragment was targeted by RT-qPCR primers of the third RT-qPCR kit. However, due to commercial confidentiality, we were unable to obtain the detection targets of the first two RT-qPCR kits, which may have contributed to the discrepancy in the results, and therefore, we advocate that the RT-qPCR method should be optimised or multiple kits should be used for the test at the same time to make its diagnostic results more sensitive and accurate.

According to the published data, the number of rabies cases has declined significantly in China year by year. But the regional distribution of rabies has not shrunk at district and county levels [6]. Furthermore, the dog population in China was about 136,109 per thousand people in 2022 [22], with many issues such as free roaming dogs and lack of dog vaccination awareness [23]. In 2020, rabies infection mainly affected the age group of 40–70 years, corresponding reported cases accounted for 69% of all cases [23]. Rabies is preventable with timely wound treatment, vaccine and administration of immunoglobulin. However, post-exposure prophylaxis vaccination rates vary greatly in different regions of China. In this study, the patient, 49-year-old, lived in a county in Anhui province. The patient might have come in contact with a dog, but did not have post-exposure prophylaxis. Anhui province belongs to medium risk groups, and 12 cases were reported in 2021 [6]. To avoid further similar tragedies in Anhui, people need to pay attention to animal contacts.

There is no effective therapy for rabies except vaccines. However, the first and only recovery case was reported in 2005 [24]. Willoughby and colleagues cured the patient by suppressing her brain function and activate her immune response. The whole process needs high experienced multidiscipline clinicians and early diagnosis, these essentials may help to control and better treat human rabies in the future. Therefore, to achieve 0 death by rabies by 2030, further "One Health" education and public advocacy should be promoted in China, including strategies directed to mass dog vaccination campaigns and population management [23].

Conclusion

The manifestations of rabies exhibit significant variation during the onset period, and nonspecific early symptoms, rapid disease progression, and an unclear exposure history impose challenges on the diagnosis, intervention, and treatment of rabies cases. Utilizing appropriate molecular testing tools, such as mNGS is imperative for rapidly confirming cases of rabies. It is crucial to enhance knowledge and awareness regarding rabies while providing suitable post-exposure prophylaxis to mitigate the incidence of human rabies and associated fatalities.

Abbreviations

RABV	Rabies virus
mNGS	Metagenomic next-generation sequencing
RT-qPCR	Reverse transcriptase quantitative polymerase chain reaction
CDC	Center for Disease Control and Prevention
ICU	Intensive care unit
CSF	Cerebrospinal fluid
bp	Base pairs
IgG	Immunoglobulin G

Supplementary Information

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Supplementary Material 1

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Not applicable.

Author contributions

Epidemiological investigation and medical information: Y. Yang, X. Zhang. Sequencing analysis: L. Liu, J. Chen, W. Zhang. Writing and original draft preparation: J. Wu, Y. Qi, Y. Shi. X. Liu Supervision: J. Wu, Y. Shi. Commenting and editing: all authors.

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Data availability

Data is provided within the manuscript or supplementary information files.

Declarations

Ethics approval and consent to participate

This study was approved and performed according to the guidelines of the Medical Research Ethics Committee of the First Affiliated Hospital of University of Science and Technology of China (Anhui Provincial Hospital) (No.2024-RE-63).

Consent for publication

Written informed consent was obtained from the patient's family for publication of this case report and any accompanying images. A copy of the written consent is available for review by the Editor of this journal.

Competing interests

The authors declare no competing interests.

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