

Original Article



Identification of Animal Rabies in Inner Mongolia and Analysis of the Etiologic Characteristics*

YIN Jing Feng^{1,†}, WANG Jin Ling^{1,†}, TANG Qing², DING Yu Lin¹, TAO XiaoYan², LI Hao², SONG Miao², GUO ZhenYang², SHEN Xin Xin², LIANG Guo Dong³, and WANG Feng Long^{1,#}

1. College of Veterinary Medicine, Inner Mongolia Agricultural University, Huhhot, 010018, Inner Mongolia, China; 2. Key Laboratory for Medical Virology, Ministry of Health, Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention (IVDC, China CDC), Beijing 102206, China; 3. State Key Laboratory for Infectious Disease Prevention and Control, Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention (IVDC, China CDC), Beijing 102206, China

Abstract

Objective To perform pathological observation and etiological identification of specimens collected from dairy cows, beef cattle and dogs which were suspected of rabies in Inner Mongolia in 2011, and analyze their etiological characteristics.

Methods Pathological observation was conducted on the brain specimens of three infected animals with Hematoxylin-Eosin staining, followed by confirmation using immunofluorescence and nested RT-PCR methods. Finally, phylogenetic analysis was conducted using the virus N gene sequence amplified from three specimens.

Results Eosinophilic and cytoplasmic inclusion bodies were seen in neuronal cells of the CNS; and rabies non-characteristic histopathological changes were also detected in the CNS. The three brain specimens were detected positive. N gene nucleotide sequence of these three isolates showed distinct sequence identity, therefore they fell into different groups in the phylogenetic analysis. N gene in the cow and dog had higher homology with that in Hebei isolate, but that in the beef cattle had higher homology with that in Mongolian lupine isolate and Russian red fox isolate.

Conclusion Rabies were observed in the dairy cow, beef cattle and canine in the farm in Inner Mongolia, in 2011, which led to a different etiologic characteristics of the epidemic situation.

Key words: Rabies; Cow; Histopathology; Phylogeny

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#Correspondence should be addressed to WANG Feng Long, Tel: 86-10-58900895, E-mail: wangfengloo@sohu.com

†These authors contributed equally to this work.

Biographical notes of the first authors: YIN Jing Feng, male, born in 1984, PhD, majoring in animal-borne disease and immunopathology; WANG Jin Ling, male, born in 1977, lecturer and PhD, majoring in animal-borne disease and immunopathology.

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INTRODUCTION

Rabies is a fatal anthroponosis mainly caused by rabies virus^[1,3,29]. More than 50 000 people and millions of animals die due to the rabies virus infection each year worldwide and nearly ten million people have received post-exposure prevention and treatment^[12,17,20]. Thousands of human cases have been reported annually in China in recent years, second to India^[27-28]. Although almost all warm-blooded animals (such as cattle and sheep) are susceptible to rabies virus, the most susceptible animals in nature are canines and felines^[21,28], as well as a number of chiroptera (like bat) and some rodents. Wild animals such as wolf, fox, and ferret badger are considered the main natural reservoir hosts of the RABV^[7]. The Inner Mongolia Autonomous Region, located in the northern frontier of China, is a main animal husbandry province of this country, whose capital, Hohhot, is known as 'Chinese milk capital'. For many years, the Inner Mongolia Autonomous Region only had experienced occasional human rabies cases, which increased to 12 in 2011^[13]. Meanwhile, rabies-like cases occurred suddenly in the dairy farm in Inner Mongolia in the same year. In an attempt to understand the underlying mechanisms for rabies infection in domesticated animals and to better prevent and treat infected human cases, we studied the brain tissue specimen of the infected cow with histopathological method to clarify its cause and characteristics. The etiologic identification, examination and analysis were also performed to verify the disease source and provide guidelines to prevent the development and spread of the rabies.

MATERIALS AND METHODS

Collection of Epidemiological Data

Annual human rabies cases of Inner Mongolia were collected from the National Surveillance Program run by the Chinese Center for Disease Control

and Prevention .

Specimen Information for Suspected Rabies Infected Animals

From February to the end of 2011, the brain tissue specimens of two cattle and one dog with suspected rabies were collected from two dairy farms and one grazing area in Da Heihe, Zhuozi Mountain and Hushi in Inner Mongolia Autonomous Region, and the background information about the specimens is shown in Table 1.

Histopathological Detection of Brain Tissue Specimens

The tissue including the cerebellum, cerebrum, hypothalamus, hippocampus, and medulla oblongata fixed in 10% neutral formalin solution was embedded with paraffin, sliced (thickness 3 μ m), plated on prevent-dull polish glass slides and dried at 50 °C for 5 h. Hematoxylin-Eosin (HE) staining was performed and the slides were observed, recorded and photographed under an optical microscope.

Rabies Specific Antigen Detection of the Brain Tissue Specimens

The three brain tissue samples were inspected with fluorescein-labeled anti-rabies virus nucleocapsid protein monoclonal antibody^[17] (CHEMICON, Rabies DFA Reagent) for direct immunofluorescence assay (DFA). The total cellular RNA of the positive and suspected positive samples detected by DFA was extracted with Trizol reagent (Invitrogen), cDNA obtained with the Ready-To-Go You-Prime First-Strand Beads kit (Amersham Bioscience), and the nest RT-PCR was performed using cDNA as the template.

Amplification and Sequencing of Rabies Virus N Gene in the Brain Specimens

The N gene coding region downstream 720 bp nucleotide sequence (corresponding to 704-1 423 region of rabies virus PV strain genome sequence M1

Table 1. Background Information of Cattle and Dog Brain Tissue Specimen of Suspected Rabies from Inner Mongolia in 2011

Specimen No.	Species	Age (years)	Number	Specimen Date	Collection Site
CNM1101C	Cow	2.5	1	2011.02.21	Daheihe
CNM1103C	Beef cattle	2.0	1	2011.08.18	Zhuozishan
CNM1104D	Dog	3.0	1	2011.11.02	Hohhot

3215) in 3 positive specimens were amplified using N644_F (5'AAGATGTGYGCYAAAYTGGAG3', 644-663) and N1537_R (5'GGATTGACRAAGATCTTGCTCAT 3', 1 515-1 537) as the primers; the PCR product was bidirectionally sequenced^[6] with the ABI PRISM 3 100 DNA sequencer (Applied Biosystems, Foster City, CA, USA) after purification (QIAquick PCR Purification Kit, QIAGEN).

Rabies Virus N Gene Nucleotide Sequence Analysis of the Brain Specimens

Besides those determined N gene sequences, other reference sequences were retrieved from Genbank. ClustalX version 1.8^[23] was applied for the full sequence alignment and MegAlign in DNASTar package (version 5.01, Laser gene, USA) was used for the sequence identity analysis of both the nucleotide and deduced amino acid sequence. The Neighbour-Joining (NJ) method with 1 000 bootstrap replications was applied to construct a phylogenetic tree using MEGA5 (5 version)^[24] and the bootstrap value of 70% was used in the phylogenetic tree^[10].

RESULTS

Epidemiological Analysis of Rabies in the Inner Mongolia Autonomous Region

The Inner Mongolia Autonomous Region experienced low numbers of rabies infections in humans in the past years^[13], with a total of 56 human rabies cases from 1996 to 2006, the cases occurring in each year except 1998 and 2005. From the epidemiological data, human rabies deaths in this Region have been gradually increasing since 2008, with 2 cases reported in 2008, 5 in 2009, 6 in 2010, and 12 in 2011, which was the highest historically. An overview of human rabies cases in the Inner Mongolia Autonomous Region in 1996-2011 is shown in Figure 1.

Fatal cases in domestic animals occurred in 2 farms and 1 grazing area in Inner Mongolia, China, in 2011. The disease was initially suspected of being pseudorabies and/or listeria disease according to the farmer's description, local epidemiological data and the infected cow's clinical symptoms. The brain tissue specimens of the cattle and dog fixed in 10% neutral formalin solution were subjected to histopathological examination^[17,29], and HE staining found eosinophilic inclusion bodies in the cytoplasm of the neurocyte^[17-18], and produced the first diagnosis of rabies. DFA, the gold standard for the

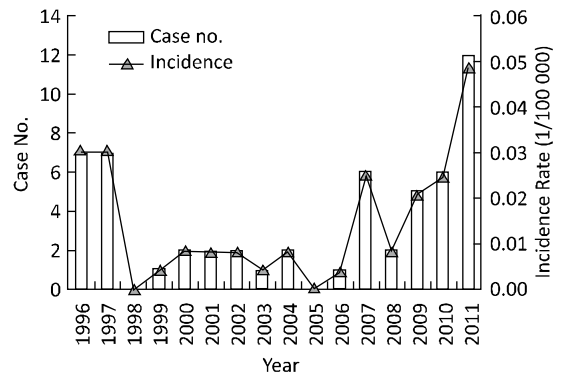


Figure 1. Overview of human rabies cases in Inner Mongolia Autonomous Region in 1996-2011.

rabies virus detection recommended by WHO, and nested RT-PCR were performed to further identify the cause and confirm the diagnosis of rabies. The research group focused on these three cases with comprehensive and in-depth research and analysis as described in the following section.

Clinical Symptoms of Infected Animals with Suspected Rabies

The period of time during which the dairy cow displayed signs of rabies was seven days, while that for the beef cattle and dog was 13 days and 5 days, respectively. The main symptoms included salivation, lacrimation, red eye, conjunctival cyanosis and painful howling. The infected dairy cows were overly agitated and active; they displayed aggressive behavior towards people, other cows, as well as walls and mechanical equipment; and were particularly sensitive to external stimuli. The infected beef cattle appeared to alternate between depressed and excited states. The advanced cases in beef cattle had hind limb paralysis, full paralysis, opisthotonos, dyspnea, masseter muscle paralysis, extended tongue and difficulty in swallowing. The infected dog was mainly excited with an abnormal appetite and tried to bite people and other animals.

Histopathological Changes of the Brain Tissue of the Infected Animals

Rabies Virus Inclusion Bodies Observation The rabies virus inclusion bodies were observed around the nucleus, in the cytoplasm, axons and dendrites in the cerebellum, cerebrum, hypothalamus, hippocampus and medulla oblongata in 2 cattles and 1 dog (Figure 2a-f). These bodies were eosinophilic, with round, oval or irregular shape and various sizes,

with diameters in the range 2-8 μm . Some inclusion bodies could push the nucleus to one side and one or several inclusion bodies could be observed in the cytoplasm in some infected neurocytes. The inclusion bodies in the axons or dendrites had a longer shape. The inclusion bodies with larger volumes contained different sizes and various numbers of vacuoles (Figure 2a).

Degenerative Change of the Neurocyte The infected neurocytes in the brain tissue of 2 cattles and 1 dog sometimes could be observed by a swollen cellular body, poorly defined or destroyed nucleolus, undefined nucleolus and mesh or impaired cytoplasm (Figure 3c). The brain tissue had significant congestion and edema. The glial cells were significantly proliferated, with some proliferated glial cells gathering around the neurocyte to form a typical satellite phenomenon. Some glial cells had absorbed the necrotic neurocyte

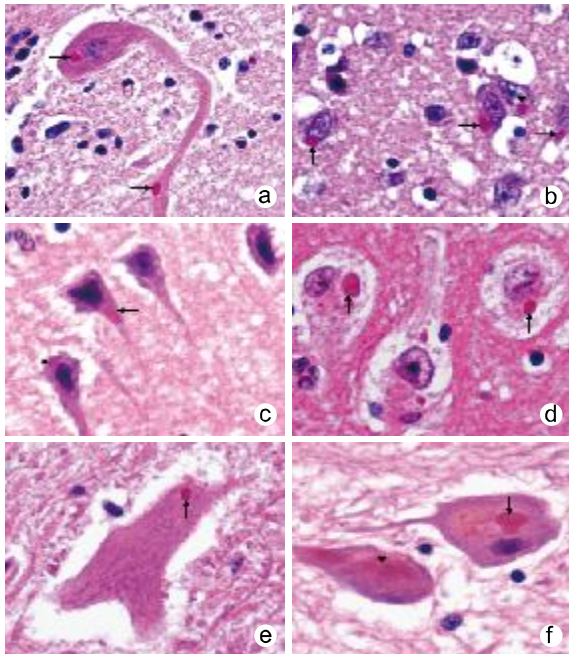


Figure 2. Histopathological changes of the brain tissue of the infected animals (a-f). The cytoplasmic eosinophilic rabies viral inclusion bodies around the nucleus and in the dendrites in the canine cerebellum Purkinje's cell (arrows, a), the cow cerebrum neurocyte (arrows, b), the cow pyramidal cell (arrows, c), the canine hippocampal astrocytes (arrows, d), the multipolar neurons in the canine medulla oblongata (arrows, e) and the canine hypothalamus (arrows, f), HE \times 400.

to display neuronophagia phenomenon. Other glial cells had proliferated in the softened and necrotic region in the nerve tissue to form glial nodules to repair or fill damaged tissue (Figure 3c).

Perivascular Cellular Infiltration The infected cow displaying rabies symptoms for 7 days had the typical lesion of non-suppurative encephalitis: vasodilation, congestion, edema, infiltration of lymphocytes and monocytes around the vessel and the proliferation of vascular adventitial cells, tissue cells and glial cells, thereby forming a perivascular cuffing composed of cells around the vessels, referred to as perivascular cuffing phenomenon or perivascular infiltration (Figure 3a, d).

Granuloma-like Lesions The brain tissue of the 2 cattles and the dog sometimes showed unclear boundaries between visible lesions and the surrounding tissue, mainly with the varying degrees of infiltration of macrophage and lymphocyte, and granulomatous-like lesions (Figure 3c).

Cavernous Lesions Interstitial vacuoles around the neuron were found in the brain tissue of the infected cow are called cerebral cavernous lesions, and give the tissue a spongy or honeycomb-like appearance (Figure 3b).

Degeneration, Dissolution, Necrosis, and Disintegration of Neurocyte The degeneration, dissolution, necrosis and disintegration of most neurocytes were observed in the brain tissue of some of the infected beef cattle, where some neurocytes appeared swollen, lightly stained or nearly disintegrated; and some appeared shrunken,

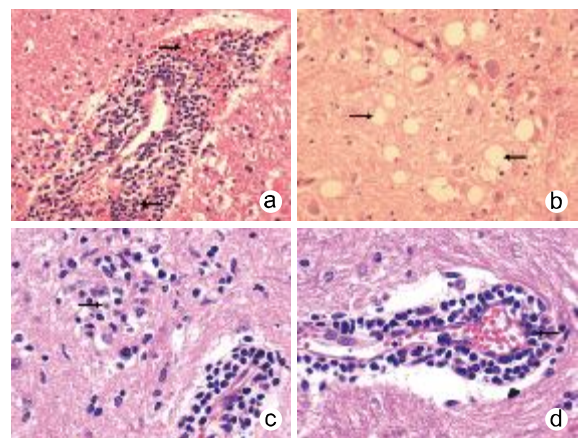


Figure 3. Cerebellum, cerebrum, hypothalamus and hippocampus showing non-characteristic histopathological changes by HE staining in the cow, beef cattle and dog with rabies (a-d), HE \times 400.

deeply stained or purple red in the nucleus and cytoplasm due to indistinct boundary between them (Figure 3c).

Hemorrhagic Lesions Hemorrhage spots can sometimes be observed around small vessels in the brain tissue in infected beef cattle (Figure 3a).

Rabies-specific Antigen Test Results

The brain tissue of the cow, beef cattle, and dog with rabies displayed a specific rabies pathogen positive signal with emerald green or apple green color under fluorescence microscope after immunofluorescence staining (Figure 4b). No rabies pathogen positive signal was observed in the negative control (Figure 4a). The rabies virus positive signal was observed in the cytoplasm, dendrite and axon in the cerebellum Purkinje's cell of the beef cattle (Figure 4c), the axon in the cerebellum Purkinje's cell of the cow, and the axon in the canine cerebrum neurocyte (Figure 4d).

Nest RT-PCR Amplification of Rabies Virus Nucleoprotein (N) Gene Fragment

The amplified nucleoprotein gene fragments obtained by nest RT-PCR amplification were around 250bp, which matched the expected size (Figure 5).

Phylogenetic Analysis of Rabies Virus Nucleoprotein (N) Gene

The accession numbers of Rabies Virus Nucleoprotein Gene for these specimens were designated as KC465376, KC465377, and KC465378,

respectively. The sequence comparison of the amino acid sequence deduced from the N gene nucleotide sequence showed that the amino acid sequence identity was 92.6%-100%, which was generally higher than the corresponding nucleotide sequence identity (88.9%-99.9%), suggesting that the mutation of the virus N gene nucleotide sequence of these three cases was mainly synonymous mutation^[26].

Using the rabies virus PV strain as the outgroup, 54 rabies virus N gene sequences were used to construct a phylogenetic tree, which is shown in Figure 6. Phylogenetic analysis showed that the N gene of these three isolates fell into the two distinct groups, CLADE I and II. In Clade I, isolates CNM1101C and CNM1104D grouped with isolates from other provinces, especially closely related isolates from neighboring region such as Hebei, Shanxi, and Beijing, indicating the possibility of transmission between the adjacent regions. Meanwhile isolates in this clade represent the predominant RABV strain currently circulating in China, suggestive of further geographical expansion of the dominant RABV strain (Figure 6). However, isolate CNM1103C collected from the beef cattle clustered with isolates from Mongolia and Russia and certain Chinese isolates, which corresponds to the Cosmopolitan Clade^[2,21-22]. In addition, CNM1103C showed higher sequence identity to Russian and Mongolian isolates than to Chinese isolates, demonstrating that RABV spread across the border was possible. Previous study showed the existence of an arctic-like rabies variant in Inner Mongolia, while none of these three isolates were associated with this variant. This diversity of RABV depicts a variety of RABV sources as well as the rapid spread of RABV.

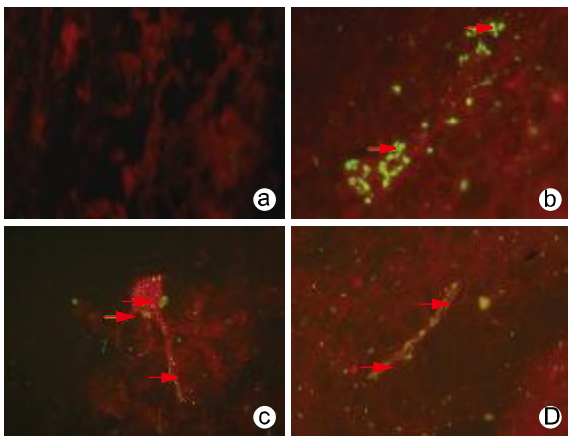


Figure 4. The specificity rabies pathogen positive signal with emerald green or apple green color in the cow, beef cattle, and dog with rabies after immunofluorescence staining (b-d). In the negative control, the rabies pathogen positive signal was not observed after immunofluorescence staining (a), $\times 400$.

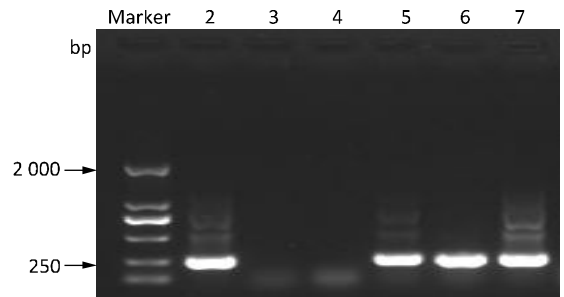


Figure 5. Electrophoresis of three samples by nest RT-PCR. lane 1: DNA Marker DL2000, lane 2: positive control, lane 3: negative control, lane 4: negative case, lane 5-7: the brain tissue of the infected cow, beef cattle and dog.

Table 2. Lyssavirus Strains Used in Study of Molecular Epidemiology of Rabies Virus in Northern China, 2011

Strains	Years	Hosts/strains	Origin	Accession No.
SC02-90	2002	Dog	Indonesia	AB154243
Hebei0(H)	2007	Homo Sapiens	China	EU267777
BeijingHu1	2009	Homo Sapiens	China	EU700031
Guizhou_Qx5	2006	Dog	China	DQ666296
Mongolia 3	2007	Wolf	Mongolia	EF614257
dog/09RS-1459/Udine/2009	2007	Cow	Mongolia	GQ478245
D664_45	-	Dog	Thailand	DQ267925
CTN	1956	Vaccine	China	AF367863
483a	-	Arctic fox	Russia	AY352487
PM1503	-	Vaccine	-	DQ099525
SRV9	-	Vaccine	China	AF499686
Eth2003	-	Wolf	Ethiopia	AY500827
3aG	1931	Vaccine	China	AF155039
DRV	2002	-	China	DQ875051
ISR-40	2000	Fox	Israel	DQ837421
3502f	-	Red fox	Russia	AY352455
-	-	Dog	India	AF374721
1294	1986	Dog	Sri Lanka	AY138549
8619NGA	1958	Bat	Nigeria	U22842
PV	1965	Vaccine	France	M13215
-	-	-	Russia	AY352459
483a	-	Arctic	Russia	AY352487
Mongolia 3	2007	Wolf	Mongolia	EF614257
MGL-22	2007	Dog	Mongolia	AB571004
-	-	-	South Korea	DQ076131
KRC5-04	2004	-	South Korea	AY730597
-	-	-	Korea	AY730595
-	-	Dog	India	AF374721
-	-	-	Indonesia	AB154243
D664_45	-	Dog	Thailand	DQ267925
01016VNM	2001	Dog	Viet Nam	EU086209
02006CBG	1998	Dog	Cambodia	EU086172
02002LAO	2002	Dog	Laos	EU086195
9915BIR	1999	Dog	Myanmar	EU086166
CTN	-	-	China	AF367863
Vaccine	-	-	China	AF499686
Vaccine	-	-	China	AF155039
Heibeio	2007	Human	China Hebei	EU267777
BD06	2006	Dog	China Hebei	EU549783
GC07	2005	2007	China Hebei	EU828655
WJ07-2	2007	Dog	China Hebei	EU828651
WJ07-1	2007	Dog	China Hebei	EU828657
BeijingHu1	2009	Homo Sapiens	China Beijing	EU70031
DRV	1989	Deer	China Jilin	DQ875051
Shanxi-BZ-6	2009	Dog	China Shanxi	GU591790
Henan_Sq17	2004	Dog	China Henan	DQ666301
Henan_Sq21	2004	Dog	China Henan	DQ666302
-	-	-	China Henan	DQ666305
Sichuan-BZ-1	2009	Dog	China Sichuan	GU591792
-	-	-	China Guizhou	DQ666296
CGZ0514D	2005	Dog	China Guizhou	EF990573
CGZ0513D	2005	Dog	China Guizhou	EF990572
CHN0525D	2005	Dog	China Hunan	EF990613

Strains	Years	Hosts/strains	Origin	Accession No.
CHN0529D	2005	Dog	China Hunan	EF990616
CHN0530D	2005	Dog	China Hunan	EF990617
CGX0612D	2006	Dog	China Guangxi	EF990587
CGX0606D	2006	Dog	China Guangxi	EF990583
CGX0604D	2006	Dog	China Guangxi	EF990581
Yunnan_Tc06	-	-	China Yunnan	EU275243
Jiangsu Wx0(H)	-	-	China Jiangsu	DQ666320
Neimeng927A	-	-	China Inner mongolia	EU284093
Neimeng927B	-	-	China Inner mongolia	EU652444
Neimeng1025B	-	-	China Inner mongolia	EU652445
Neimeng925	-	-	China Inner mongolia	FJ415313
Neimeng1025C	-	-	China Inner mongolia	EU284094

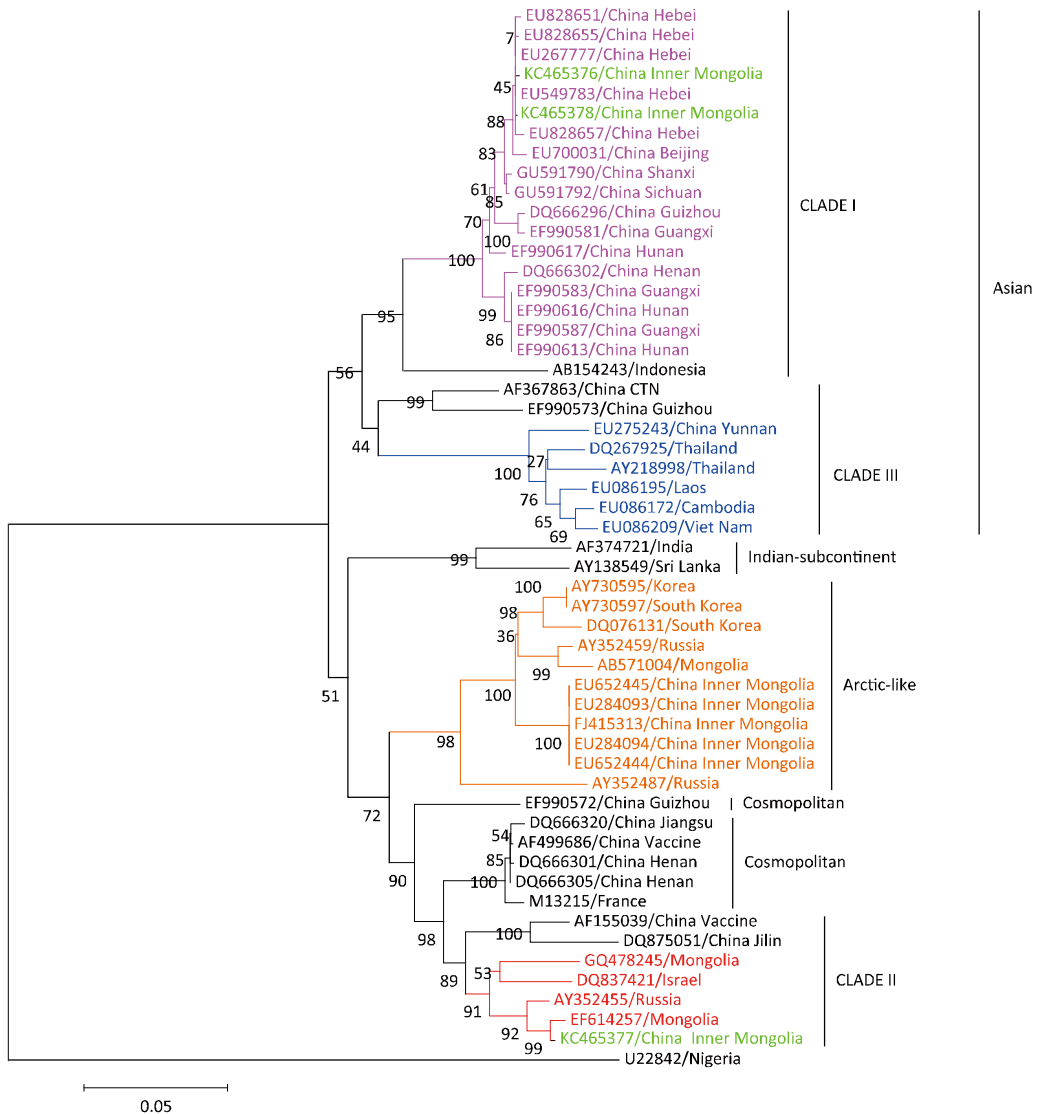


Figure 6. Neighboring-Joining Phylogenetic tree of 3 specimens of rabies virus from Inner Mongolia of China, 2011, and different genotypes (GTs) from other areas based on a 720-nt (nt 704-nt 1 423) nucleoprotein (N) gene fragment of RABV.

DISCUSSION

The eosinophilic and cytoplasmic rabies virus inclusion bodies in the neurocyte in the brain tissue^[16-17,19] could be considered as an important basis for the rabies diagnosis using traditional histopathological methods^[5,25,32]. But some reported that the rabies virus inclusion bodies were not observed in the neurocyte cytoplasm in the brain tissue of 20%-60% of the cases with rabies^[15,17,19,29]. The gold standard for laboratory rabies confirmation recommended by WHO was DFA and nest RT-PCR^[28] for the brain tissue. In this study, the brain tissues of 3 cases in Inner Mongolia were detected positive with rabies specific immunofluorescence and RT-PCR and were also confirmed as rabies infection^[18-22].

In addition to the characteristic rabies virus eosinophilic and cytoplasmic inclusion bodies^[8], other histopathologic changes were observed in the neurocyte cytoplasm in the brain tissue^[5,12-13,29]. The infiltration of lymphocytes and monocytes around the vessels in the brainstem and the formation of the perivascular 'cuffing' phenomenon were observed^[12,16,29]. The haemorrhage appeared around small vessels, so did the cerebrum interstitial vacuolization, proliferation of glial cells in the softening and necrotic region of the nerve tissue to form glial nodules, degeneration, dissolution, necrosis and disintegration of some neurocytes (Figure 3a-b). The swollen bodies, unclear or disappeared nucleus, unclear nucleolus and mesh or impaired cytoplasm in the infected neurocytes in some brain tissue were located. An indistinct boundary between the visible lesions and the surrounding tissue in the cow brain tissue with different infiltration of macrophage and lymphocyte and granulomatous lesions was present (Figure 3c), which was consistent with the granuloma-like lesions in the canine brain tissue with rabies detected with immunohistochemical technology by Kipar et al. (1998). Moreover, the rabies virus was also evaluated to transfer between neurons along with the dendrite or axon with histopathological observation and immunohistochemical fluorescence (Figure 4b, c, d), which was consistent with the results reported by Minoru Tobiume and Hu Rong-liang^[16,25].

By phylogenetic analysis of rabies virus (Figure 6), we clearly demonstrated that the viral N gene of the cow and dog belonged to the same branch,

which were separated from the beef cattle, indicating that cow and dog virus possessed closer relationship (Figure 6). In combination with the geographical condition of the dog with rabies occurred near the farm, the dog rabies may be epidemically related to the cow's at the genetic level, however, we could not exclude that the region might have different virus sources.

The homologous comparison of amino acid sequence deduced from the N gene nucleotide sequence showed that the amino acid sequence displayed 92.6%-100% homology of these three cases, which was generally higher than the corresponding nucleotide sequence homology (88.9%-99.9%), showing that the mutation of the virus N gene nucleotide sequence of three cases in Inner Mongolia in 2011 was mainly synonymous mutation^[26].

In addition, the virus N genes of the cow, dog, Hebei isolates [Hebei0 (H), GC07, BD06, WJ07-2, and WJ07-1], Beijing isolate (Beijing Hu 1) and Shanxi canine isolate (Shanxi-BZ-6) all belonged to the same branch CLADE I (Figure 6), whose N gene nucleotide sequence similarity between the cow and Hebei0 (H), BeijingHu1 and Shanxi-BZ-6 reached 99.6%, 99.3%, and 99.5%, respectively. The nucleotide sequence similarity was the same as with dog, showing a close origin of the virus. Combined with the geographical map, the rabies virus may have the similar spread trend from Beijing, Hebei, and Shanxi to Inner Mongolia.

The virus N gene of the beef cattle had high homology with that of the Mongolian lupine isolate-Mongolia 3. Mongolian cows isolate dog/09RS-1459/Udine/2009 and Russian red fox isolate 3502f (>99%) all were categorized into the same branch CLADE II, showing a close relationship between them. In combination with the geographical conditions among northern China, Mongolia, Russia and other countries, the beef cattle, Mongolia3, dog/09RS-1459/Udine/2009 and 3502f might have the same virus source suggesting that the beef cattle virus might derive from Mongolia or Russia^[9]. Additionally, the branch CLADE II that the beef cattle virus belongs to is clearly separated from the branch Arctic-like of other strains in Inner Mongolia (NeiMeng927A, NeiMeng927B, NeiMeng1025B, NeiMeng925, and NeiMeng1025C). Another rabies branch, CLADE I from cow and dog, possesses distant relationship^[31,33]. Overall, Inner Mongolia may have three different sources of the rabies virus.

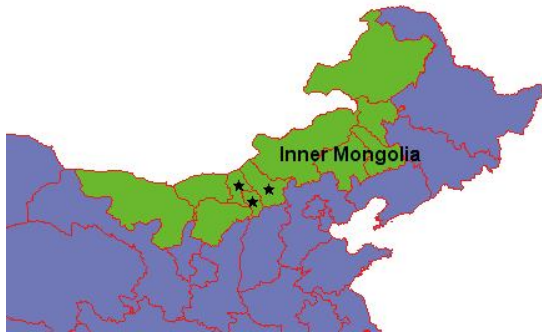


Figure 7. The region where confirmed cases with rabies were discovered in Inner Mongolia, China, is labeled with a black star.

The Inner Mongolia Autonomous Region was a region with incidental or few reports of rabies among the residents in the past^[13]. However, the number of people with rabies increased to 12 in 2011^[13], which was in step with the increase in the number of animals (such as dairy cow, beef cattle, dog, and raccoon) with rabies in 2011. Therefore, the increase of the people suffering from rabies in Inner Mongolia Autonomous Region in 2011 was closely associated with the increase of the animal with rabies.

The cases of rabies were all discovered in the central and western areas of Inner Mongolia, situated in north to the Yellow River (Figure 7), and no case was found in the south area of the Yellow River, which indicates that the Yellow River serves as the natural barrier to the dogs and wild animals (such as wolf, fox, and ferret badger)^[26].

It is well known that the dogs are the main hosts and infectors of rabies^[7] in developing countries in Asia and Africa. Of the human deaths from rabies 99% were caused by dog biting^[20-21,28]. Interestingly, the infection of domestic animals with rabies was also mostly caused by dog biting. Rabies transmitted by dog biting has a great impact on the local economy and public health in Latin America^[28]. In Brazil, it was estimated that the annual number of rabies infections in cattle caused by dog biting was more than 842 000, which posed a huge threat to the people in proximity to livestock^[7]. According to Inner Mongolia herdsmen's description, rabies appeared on the farms soon after the arrival of infected dogs, which showed that the dog was the suspected animal spreading the rabies in the cattle farm. On the contrary, rabies could be effectively controlled after strictly preventing the dog from entering the farm, further indicating that the dog is

the villain to spread the rabies in the cattle farm. Thus, enhancing the management and immunization of the dogs is essential for preventing rabies spreading between humans and animals.

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