

## SHORT REPORT

### An outbreak of pig rabies in Hunan province, China

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#### SUMMARY

The first dog-associated outbreak of rabies in swine in China (Hunan province) has been diagnosed and the related virus isolated. Sequence analysis showed that the pig isolate was a genotype 1 rabies virus with a very high nucleotide identity to local dog isolates.

On 11 February 2006, frozen brain specimens of two pigs suspected of dying from rabies were received in our laboratory at the Military Veterinary Research Institute (MVRI), Academy of Military Medical Sciences for confirmation. They originated from a rural pig farm in Yongzhou city, Hunan province. The specimens were tested by the fluorescent antibody test (FAT), mouse inoculation test (MIT) and RT-PCR. The FAT and MIT procedures were based on the WHO protocols [1] using brain smears and FITC-conjugated anti-rabies monoclonal antibody (made in our laboratory) for the FAT. Results showed that the two brain tissues were positive for rabies antigen. A litter of suckling mice inoculated intracranially with the brain tissue suspensions died of rabies 15–17 days post inoculation. Brain smear from each dead mouse was positive for rabies virus (RABV) by the FAT method. The pig rabies virus isolate was designated HuNPN01. For testing by RT-PCR, total RNA was extracted from

homogenates of the two pig brain tissues by TRIzol<sup>®</sup> (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, and reverse transcription (RT) was carried out using a mixture of 6-mer random primers, oligo-d(T)<sub>15</sub> and M-MuLV reverse transcriptase RNase H<sup>-</sup> (New England Biolabs Inc., Ipswich, MA, USA). The RT product was used to amplify almost the full *N* gene of rabies virus with an expected size of 1353 bp using Ex-Taq DNA polymerase (Takara Ltd, Dalian, Liaoning, China) and two primers (forward NF: 5'-GTCGAATTC-ATGGATGCCGACAAG ATTGTATTCAAAG-3' and reverse NR: 5'-GTAGGATCCTGAGTCA-CTCGAATATGTCTTGTTTAG-3'). The forward primer and the reverse primer match with 28 nt and 30 nt of the *N* gene respectively. The PCR was performed in a Peltier Thermal Cycler PTC-200 (Bio-Rad DNA Engine<sup>®</sup>, Hercules, CA, USA) under the following conditions, 94 °C for 2 min, then 35 cycles of 94 °C for 2 min, 60 °C for 30 s, 72 °C for 60 s, and finally 72 °C for 10 min, followed by storage at 4 °C. Both specimens demonstrated clear amplification of a 1353 bp amplicon of the RABV *N* gene (data not shown). The PCR amplicon of brain tissue of the isolate HuNPN01 was cloned into pMD18-T vector

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and subsequently sequenced by a commercial sequencing service (Takara Ltd). The sequence, including the two matching primers, has been deposited in GenBank (accession number DQ496219).

The Yongzhou farmer neither reported the outbreak to the official veterinary administration at its start, nor took any measures to halt the disease during its course. The outbreak was almost over when he sent the two heads (one dead from the disease and the other killed when moribund) to our local laboratory at the Veterinary College of Hunan Agricultural University on 9 February, 4 days before the last pig death on 13 February. After laboratory confirmation that both samples were positive for RABV, a retrospective epidemiological investigation was carried out by visiting the affected farm. The farm originally contained 56 fattening pigs weighing about 50 kg, raised in four pens linked together in a line. On 19 December 2005, the farmer's neighbour's dog was found to be acting strangely, roaming around pen 2 and leaving a bleeding bite on the snout of a pig through the pen wall bars. Twenty days later, on 8 January 2006, the injured pig in pen 2 developed a furious disease (referred to as the index case) and started to bite other pigs in the same group ( $n = 16$ , the farmer could not remember clearly the location or severity of the pig bites). The index pig died 2 days after developing rabies symptoms. Although the farmer was unable to remember accurately the dates and sequence of each onset of the subsequent cases, it appears that during the next few days, two of the pigs bitten by the index pig in pen 2 developed the disease. The cases increased in the pen at the rate of 1–2 cases per day with a few furiously rabid pigs jumping the pen walls to bite pigs in the two adjoining pens. About 12 days later an onset of the disease was noticed in pens 1 and 3, indicating that the incubation period of the disease was about 10 days. The farmer reported that the disease was not present in pen 4 since no affected pig jumped into this pen. Almost all infected pigs developed furious rabies except the last one, which died suddenly on 13 February without any symptoms. The clinical manifestations included hyperexcitation, roaring and attacks on other pigs within the herd. Some affected pigs exhibited spasms in response to tactile, auditory and visual stimuli when people visited and when water was poured into their drinking tanks. All deaths occurred 2–3 days following the appearance of symptoms, with some carcasses being almost destroyed by the bites of rabid pigs. The outbreak lasted 35 days from 8 January

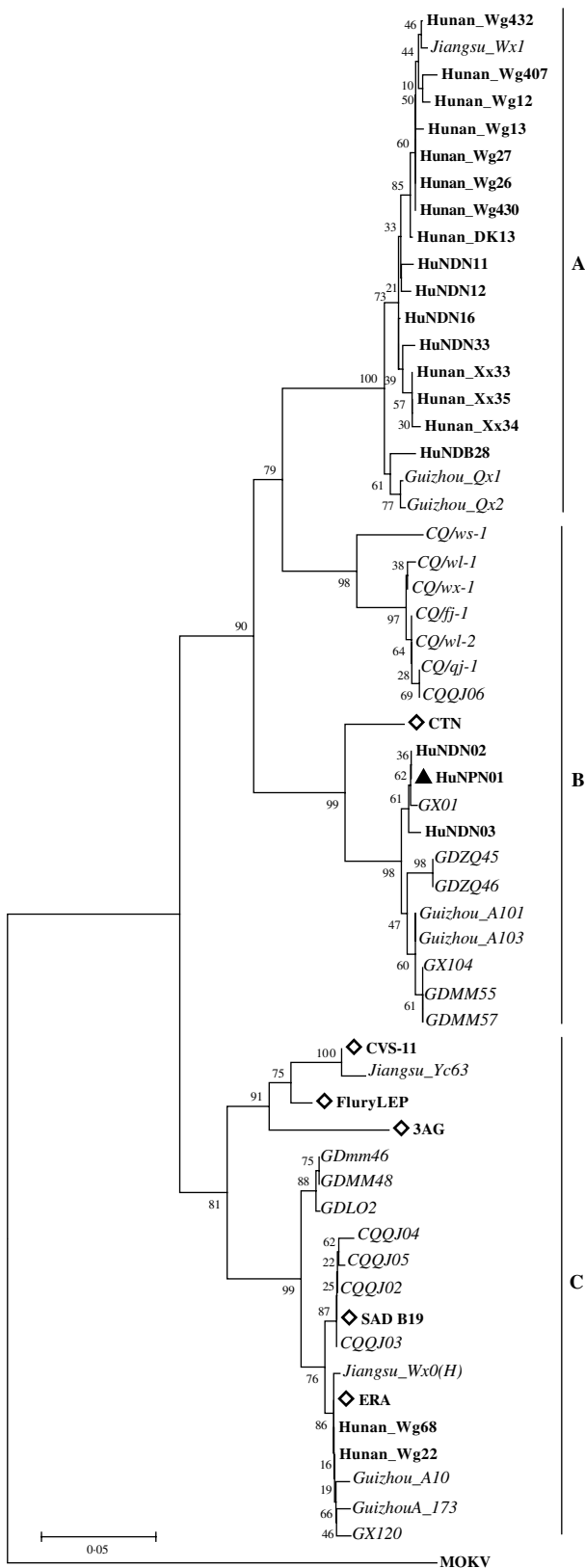
to 13 February 2006, and resulted in 20 pig deaths including one culled when moribund. Twelve cases occurred in pen 2, and eight in the two adjoining pens. The remaining 36 pigs remained healthy and gained weight to around 70–80 kg by the end of March. During the outbreak no control measures were applied, and therefore the spread of the disease within the herds was not stopped by destruction of the first cases.

Although documented in textbooks [2], rabies in swine is uncommon and has been poorly described [3–9]. It accounts for only 0.1–1.1% of the incidence of animal rabies [4, 6, 8]. The outbreak described here, causing the death of 20 out of 56 pigs is the first case report of pig rabies in China, featuring the acute and furious forms with an incubation time of 20 days for the index pig and about 10 days for contact animals. The incubation period observed in the present study is consistent with that observed by Luangtongkum *et al.*, who reported that the incubation period of three lethal pig rabies infections caused by dog bites is 8–16 days [5]. After the appearance of symptoms, all affected pigs died within 1–3 days and showed furious clinical symptoms similar to those described previously [4–6, 8, 9], although an outbreak of this size has not been reported so far. This demonstrates that rabies can be transmitted naturally among pigs, and that swine are highly susceptible to rabies, with the disease readily spreading in pig herds if culling is not carried out at an early stage.

Rabies is an almost invariably fatal zoonotic viral disease that can affect all warm-blooded animals worldwide. Infection following dog bites accounts for China having the second highest number of human rabies cases in the world [10]. Hunan is one of the most severely affected provinces in the country, with about 400 human rabies deaths annually [11, 12]. Yongzhou is a district comprising eight counties and one city of that name. Due to the high number of free-roaming dogs, which are raised for security purposes and rarely vaccinated, this district has experienced the most severe rabies epidemics in Hunan province. From 2000–2003 the annual incidence of human rabies cases in Yongzhou district was 47, 81, 75 and 108 respectively, the highest in the province and accounting for 25% of the total provincial cases [12]. In 2005 and 2006, the corresponding numbers were 36 and 64 respectively (unpublished data, Hunan Provincial Center of Disease Control). There is, however, a lack of information about the incidence of animal rabies in the district. The rabies-affected pig

Table. *The 57 strains or isolates of rabies viruses used in the study except for pig isolate HuNPN01*

Strain	Source	Province	Accession no.	Reference
3AG	Vaccine	Beijing	AF155039	Unpublished
CTN	Vaccine	Beijing	AF367863	Unpublished
CVS-11	Human		AB069973	Unpublished
SAD B19	Vaccine		M31046	[14]
ERA	Vaccine		EF206707	Unpublished
Flury LEP	Vaccine		DQ099524	Unpublished
MOKV	Shrew		Y09762	[15]
Hunan_Wg12	Dog	Hunan	DQ666308	[13]
Hunan_Wg13	Dog	Hunan	DQ666309	[13]
Hunan_Wg22	Dog	Hunan	DQ666310	[13]
Hunan_Wg26	Dog	Hunan	DQ666311	[13]
Hunan_Wg27	Dog	Hunan	DQ666312	[13]
Hunan_Wg68	Dog	Hunan	DQ666313	[13]
Hunan_Wg407	Dog	Hunan	DQ666314	[13]
Hunan_Wg430	Dog	Hunan	DQ666315	[13]
Hunan_Wg432	Dog	Hunan	DQ666316	[13]
Hunan_Xx33	Dog	Hunan	DQ666317	[13]
Hunan_Xx34	Dog	Hunan	DQ666318	[13]
Hunan_Xx35	Dog	Hunan	DQ666319	[13]
Hunan_DK13	Dog	Hunan	DQ666307	[13]
HuNDN02	Dog	Hunan	DQ515994	Unpublished
HuNDN03	Dog	Hunan	Submitted	Unpublished
HuNDN11	Dog	Hunan	DQ515995	Unpublished
HuNDN12	Dog	Hunan	DQ515996	Unpublished
HuNDN16	Dog	Hunan	DQ515993	Unpublished
HuNDN28	Dog	Hunan	Submitted	Unpublished
HuNDN33	Dog	Hunan	Submitted	Unpublished
CQ/fj-1	Dog	Chongqing	DQ108987	[16]
CQ/qj-1	Dog	Chongqing	DQ108986	[16]
CQ/wl-1	Dog	Chongqing	DQ108989	[16]
CQ/ws-1	Dog	Chongqing	DQ108988	[16]
CQ/wx-1	Dog	Chongqing	DQ108985	[16]
CQ/wl-2	Dog	Chongqing	Submitted	[16]
CQQJ02	Dog	Chongqing	Submitted	Unpublished
CQQJ03	Dog	Chongqing	Submitted	Unpublished
CQQJ04	Dog	Chongqing	Submitted	Unpublished
CQQJ05	Dog	Chongqing	Submitted	Unpublished
CQQJ06	Dog	Chongqing	Submitted	Unpublished
Guizhou_A10	Human	Guizhou	DQ666288	[13]
Guizhou_Qx2	Dog	Guizhou	DQ666295	[13]
Guizhou_A103	Dog	Guizhou	DQ666290	[13]
Guizhou_Qx1	Dog	Guizhou	DQ666294	[13]
Guizhou_A101	Dog	Guizhou	DQ666289	[13]
Guizhou_A173	Dog	Guizhou	DQ666293	[13]
Jiangsu_Wx1	Dog	Jiangsu	DQ666321	[13]
Jiangsu_Wx0(H)	Human	Jiangsu	DQ666320	[13]
Jiangsu_Yc63	Dog	Jiangsu	DQ666322	[13]
GDZQ45	Dog	Guangdong	Submitted	Unpublished
GDZQ46	Dog	Guangdong	Submitted	Unpublished
GDMM55	Dog	Guangdong	Submitted	Unpublished
GDMM57	Dog	Guangdong	Submitted	Unpublished
GDMM46	Dog	Guangdong	Submitted	Unpublished
GDMM48	Dog	Guangdong	Submitted	Unpublished
GDL2	Dog	Guangdong	Submitted	Unpublished
GX01	Dog	Guangxi	DQ866105	Unpublished
GX120	Dog	Guangxi	DQ866089	Unpublished
GX014	Dog	Guangxi	DQ866106	Unpublished



**Fig.** The 371 bp sequence of the *N* gene 5'-end was used for phylogenetic analysis within genotype I, which was conducted using MEGA version 3.1 (<http://www.megasoftware.net/>) (using the neighbour-joining algorithm) and PHYLIP

farm is located in a village in the southwest suburbs of Yongzhou city, where no human rabies has been reported in recent years.

The most recent study has shown that the viruses causing rabies in China are genotype I strains consisting of three genetic subgroups [13]. The dog responsible for the pig outbreak escaped and was found dead near the house of its owner the next day after biting the index pig. Unfortunately it was buried without being reported to a veterinary diagnostic agency. In order to investigate the genetic relationship of the pig isolate with dog isolates circulating in China, an *N* gene-based phylogenetic analysis focusing on genotype I rabies viruses was conducted using the sequences currently available from GenBank, which were obtained from rabid dogs identified in Hunan, Guangxi, Guangdong, Guizhou, and Jiangsu provinces, and Chongqing city (see Table). The analysis showed that the Chinese rabies viruses can be classified into three major subgroups, A, B and C, and in Hunan province isolates of all three subgroups were prevalent. The pig virus HuNPN01 was segregated into subgroup B, showing the highest genetic identity with some isolates recently obtained from dogs in Hunan (HuN) and Guanxi (GX), Guizhou (GZ) and Guangdong (GD) provinces (see Fig.). Further genetic comparison using DNASTAR software (DNASTAR Inc., Madison, WI, USA) showed that the partial *N* gene of HuNPN01 was 99.7% (370/371) identical to HuNDN02 isolated in 2005 from a rabid dog in Hengshan city near Yongzhou. Sequence comparison with four Chinese vaccine strains, CTN and 3AG for human (killed vaccine), ERA and Flury LEP for animal (live vaccine) showed that the full *N* gene sequence of HuNPN01, excluding the portions matching with primers, had 94.4% (1223/1295 nt), 86.3% (1118/1295 nt), 87.2% (1129 /1295 nt) and 87.3% (1131/1295 nt) identity respectively, indicating that the pig isolate did not originate from the widespread use of live vaccine in dogs.

version 3.63 (Seattle, WA, USA) (using the maximum parsimony method). The tree was estimated statistically by 1000 replicates of the bootstrap value and visualized by the TREEVIEW program (<http://taxonomy.zoology.gla.ac.uk/rod/treeview.html>). Genotype 3 MOKV was used as the outgroup and all sequence information is shown in the Table. The three major subgroups, A, B and C, correspond with published nomenclature [13]. **Bold type**, isolates of Hunan Province; *italic type*, isolates of other provinces; ▲, pig isolate HuNPN01; ◇, vaccine and challenge strains.

Our data strongly support the conclusion that the outbreak in swine was caused by the rabid dog. Moreover, the history of exposure to a dog bite, the rabies-like clinical signs, and the positive FAT, MIT and RT-PCR results indicate that rabies was only disease entity involved in the outbreak.

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#### DECLARATION OF INTEREST

None.

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