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# The N and P genes facilitate pathogenicity of the rabies virus G gene

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**ABSTRACT**: To explore the effects of different gene combinations on the pathogenicity of the rabies virus (RABV), six chimeric RABV mutants, rRC-HL(G), rRC-HL(NG), rRC-HL(PG), rRC-HL(NP), rRC-HL(NM) and rRC-HL(NPG), were constructed using a reverse genetic technique based on an avirulent parental rRC-HL strain and a virulent parental GX074 isolate. These mutants were intracerebrally inoculated into adult mice. The results indicated that $10^2$ ffu and $10^6$ ffu of rRC-HL(G), rRC-HL(NG), rRC-HL(PG) and rRC-HL(NPG) were 100% lethal. In the case of intramuscular viral infection, none of the mice inoculated with $10^2$ ffu of any of the RABV mutants, including GX074, died; at $10^6$ ffu, rRC-HL(G) was lethal in 2/5 cases, rRC-HL(NG) was lethal in 1/5 cases and rRC-HL(PG) was lethal for 2/5 mice. The rRC-HL(NPG) mutant was fatal for 3/5 mice, as was the parental GX074. Furthermore, the $LD_{50}$ values of the chimeric RABV mutants were measured, with the results showing that the $LD_{50}$ values of both rRC-HL(NG) and rRC-HL(PG) were lower than that of rRC-HL(G), but higher than that of rRC-HL(NPG). Thus, the action of N + G, or P + G, or N + P + G gene combinations may be more pronounced than that of the G gene alone. Body weight changes and the clinical symptoms of the tested mice were consistent with pathogenicity. These data indicate that the N and P genes are involved in and facilitate the pathogenicity of the RABV G gene. These experiments provide further evidence that multi-gene cooperation is responsible for the virulence of RABV.

**Keywords**: reverse genetics; gene combination; chimeric virus; lethality

Rabies virus kills more than 59 000 people each year (Tan et al. 2017). Approximately 3.9 billion people in more than 150 countries are at risk, and the virus continues to represent a serious global public health threat (Yin et al. 2013). The rabies virus (RABV) possesses a single negative-stranded RNA genome that encodes five structural proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and large protein (L). The G protein plays an important role in viral pathogenicity and neuroinvasiveness. Previous studies have focused on the pathogenicity of the G protein, because the arginine or lysine residues at position 333 within the glycoprotein have been shown to be required for the virulence of fixed RABV (CVS strain) in adult mice (Dietzschold et al. 1983; Seif et al. 1985; Tuffereau et al. 1989). Moreover, mice inoculated with RABV mutants harbouring a glutamine instead of a lysine at position 147 displayed significantly decreased mortality (Prehaud et al. 1988). Investigations into RABV pathogenicity have uncovered that the amino acids at positions 242, 255 and 268 within the glycoprotein are also responsible for viral pathogenicity (Takayama-Ito et al. 2006). Although it has been suggested that RABV pathogenicity is under the control of mul-

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tigenic factors, research has mainly focused on the fixed virus (Yamada et al. 2006; Shimizu et al. 2007) and also on street virus (Faber et al. 2004; Pulmanausahakul et al. 2008); however, the pathogenesis of RABV strains derived from various animal species and regions is different.

In this study, a street RABV isolate, GX074, obtained from a clinically healthy dog in the Guangxi Province of southern China was investigated. GX074 was identified to belong to RABV Group II of Guangxi province and is known to be highly pathogenic in the adult mouse (Liu et al. 2007; Tang et al. 2014). The RC-HL strain, a fixed virus that was used for the production of an animal rabies vaccine in Japan, has been well-investigated with regard to biological security and immunogenicity. Here, the GX074 isolate was used as a model to further explore the molecular mechanism of RABV pathogenicity and was used to explore the potential multigenicity of RABV pathogenicity. Six recombinant chimeric RABV mutants containing various GX074 genes were constructed based on an infectious cDNA clone of the attenuated RC-HL strain using a reverse-genetics approach. Mouse cell lines and animal models were then inoculated with the variant viral constructs and virulence and lethality were assessed. The main purpose of these experiments was to investigate multi-gene cooperation in the pathogenicity of the rabies virus.

## MATERIAL AND METHODS

**Ethics statement**. All animal experiments were performed according to the National Guideline on the Humane Treatment of Laboratory Animals Welfare (MOST of People's Republic of China, 2006) and approved by the Animal Welfare and the Animal Experimental Ethical Committee of Guangxi University (No. Xidakezi 2000138). All husbandry and experimental procedures were conducted in compliance with the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals.

**Cells and viruses**. Mouse neuroblastoma (NA) cell lines were maintained in Dulbecco's modified eagle medium-high glucose (DMEM) supplemented with 10% foetal calf serum (FCS). BSR-T7/9 cells used to recover RABV from infectious complementary DNA (cDNA) were maintained in DMEM supplemented with 10% FCS. Cell lines were incubated at 37 °C, 5% $CO_2$. The street RABV GX074 isolate was obtained from a clinically healthy dog in the Guangxi Province of southern China (Liu et al. 2007; Tang et al. 2014). The rRC-HL strain was recovered from infectious cDNA constructed from the RC-HL strain, as previously reported (Ito et al. 2001a). All viral stocks were prepared in NA cells and stored at –80 °C. Viral titres were determined using a focus-forming assay in NA cells with the anti-N protein monoclonal antibody (MAb) 8-1 (kindly provided by Dr. Nobuyuki Minamoto, Gifu University, Japan) (Minamoto et al. 1994). All viruses were intracerebrally injected into four-week-old Kunming mice (from the Animal Center at Guangxi Medical University, Guangxi, China). The mice brains were harvested and homogenised in PBS to yield 1 : 5 (w/v) emulsions and stored at –80 °C.

**Reverse transcription PCR (RT-PCR)**. Genomic RNA from the viral stocks was extracted using TRIzol (Invitrogen, Carlsbad, USA), according to the manufacturer's instructions. Single-stranded cDNA was synthesized by reverse transcription (RT) using AMV Reverse Transcriptase (Promega, Madison, USA), according to the manufacturer's instructions. PCR was performed using the TaKaRa Ex-Tag Kits (TaKaRa, Dalian, China).

**Cloning and sequencing of cDNA fragments**. The amplified cDNA fragments were cloned into a pMD-18T vector (TaKaRa) and purified with the MiniBEST Agarose Gel DNA Extraction Kit Ver. 4.0 (TaKaRa) according to the manufacturer's instructions. Sequencing of the amplified cDNA fragments was performed by Invitrogen (Guangzhou, China).

**Construction of the infectious RABV cDNA clones and rescue of the recombinant chimeric viruses**. The pRC-HL plasmid containing the restriction enzyme site *Pst*I was kindly provided by Dr. Nobuyuki Minamoto (Gifu University, Gifu, Japan). Five infectious cDNA clones based on RC-HL and GX074 were constructed. Primers used for the construction of the RABV mutants are presented in Table 1.

Recombinant chimeric RABV mutants were rescued as described by Ito (Ito et al. 2001b). Briefly, BSR-T7/9 cells constitutively expressing bacteriophage T7RNA polymerase were grown in 24-well plates in DMEM supplemented with 10% FCS, and incubated at 37 °C, 5% $CO_2$ overnight to 80% confluence. Cells were transfected with 2.0 μg of the full-length plasmid, 0.4 μg of pIRES-N, 0.1 μg of pIRES-P and 0.2 μg of pIRES-L using Lipofectamine

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Table 1. Primers used for the construction of chimeric rabies virus mutants

| Primers | Sequences | Position (nt) | Gene |
|---|---|---|---|
| NP6 | 5'-TGCAGTCGGTCACGTGTTCAATCTC-3' | 979–1003 | N |
| RPP2 | 5'-TCGGATTGACGAAGATCTTGCTC-3' | 1517–1539 | |
| PP1 | 5'-ATGAGCAAGATCTTCGTCAATCCG-3' | 1515–1538 | M |
| RPP4 | 5'-GATGAGCCCGCGGGATATAGTCTG-3' | 3106–3130 | |
| GP3 | 5'-AGCCTCGTGGATGTGAAAAAAACTATTAACATCCCTCAAA AGACTTAGGGAAAGATGATTCCTCAAGTTCTTTTGTTTG-3' | 3262–3341 | G |
| GP4 | 5'-GATCTGCAGAACTTGAAGCGTCGAAAAGATGACAAATCT TCACAGCTTGGTCTCACCTCCGC-3' | 4867–4929 | |
| RMGP1 | 5'-CAATGTCATATCCAAGGCAGAATC-3' | 2977–3001 | P |
| RMGP2 | 5'-GTTTTTTTCACATCCACGAGGC-3' | 3264–3286 | |

2000 (Invitrogen, USA) according to the manufacturer's protocol. After 4–6 h, the transfection medium was replaced with fresh DMEM supplemented with 10% FCS. The supernatants were collected five to seven days later, transferred into wells containing NA cells and incubated for three days.

The rescued viruses were examined by an indirect fluorescence assay (IFA) using the anti-N monoclonal antibody (MAb) 8-1. The supernatants from virus-positive culture wells were collected to propagate the viral stock in the NA cells. The sequences of the recovered viruses were confirmed by sequencing the corresponding gene fragment of the viral genome. Viral titration was performed using a focus-forming assay in which the foci were counted under a fluorescence microscope (Nikon ECLIPSE Ti, Japan) and calculated as focus-forming units per milliliter (ffu/ml).

**Indirect immunofluorescence assay (IFA)**. Firstly, $2 \times 10^5$ NA cells were seed in 24-well plates in DMEM supplemented with 10% FCS and incubated at 37 °C with 5% $CO_2$. At 80% confluence, six rescued chimeric rabies viruses were incubated in NA cells for 1–2 h at 37 °C and 5% $CO_2$, and then the primary viral supernatant was removed and fresh DMEM containing 10% FCS was added; subsequently, the cells were incubated for 48–72 h. The cultures were centrifuged at 10 000 *g* at 4 °C for 10 min, and the supernatants were co-cultured with NA cells in 24-well plates. Next, the supernatant was discarded and the infected cells were fixed with acetone : methanol (1 : 1) at 4 °C for 10 min. The fixing solution was discarded and the cells were washed three times with phosphate-buffered saline (PBS) containing 0.1% Tween-20 (PBS-T). Next, the cells were incubated with the primary antibody (anti-N Mab 8-1, 1 : 4000 diluted in PBS) at 37 °C for 1 h. The primary antibody was discarded and the cells were washed three times with PBS-T and incubated with the secondary antibody (Anti-mouse IgG (whole molecule)-FITC, F9137, Sigma, 1 : 300) at 37 °C for 1 h and washed three times with PBS-T. Fluorescence was analysed using an inverted fluorescence microscope (Nikon ECLIPSE Ti, Japan).

**Virus growth in cell culture and passaging**. Because the virus titre of the F1-4 generation was too low, viruses were blindly passaged and cells were infected with 100 ul of each virus in every passage. From F5, each virus strain was used to infect cells with 0.01 MOI. Briefly, monolayer cultures of NA cells in six-well plates were infected with 100 µl or 0.01 MOI of each virus. After 1 h of virus adsorption, the inoculum was removed and the monolayer was washed three times. Fresh medium was added and the cells were incubated at 37 °C. The culture media were harvested at 72 h post-infection. The F4, F7 and F11 passage of each virus strain was titrated in NA cells using the focus assay as described above.

**RABV pathogenicity in mice**. The pathogenicity assay was performed by intracerebral injection of the virus into four-week-old Kunming mice from the Animal Center at Guangxi Medical University (Guangxi, China). Other parameters were measured after intramuscular injection of the chimeric RABV mutant viruses into four-week-old mice; DMEM (Gibco, USA) was used as a control. Each group of mice was intracerebrally or intramuscularly inoculated with 0.03 ml (diluted in DMEM) containing $10^2$ ffu or 0.1 ml 1 : 5 mice brain emulsion ($10^6$ ffu) of each strain into the left thigh muscle. The mice were observed daily, and those mice intracerebrally inoculated with $10^2$ ffu of each virus

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strain were weighed daily for 21 days. Symptoms were recorded and mice were classified into six grades based on body weight loss, neurological symptoms and survival or death. Groups of five four-week-old mice were inoculated *i.c.* with 30 µl of serial ten-fold dilutions of each virus, respectively. The median lethal dose ($LD_{50}$) of each virus was calculated by the method of Reed and Muench (Shimizu et al. 2007). The mice were euthanised in a container with halothane inhalant once they displayed a lack of righting reflex (mice unable to right themselves within 10 s after being placed on their side).

## RESULTS

### Construction of infectious cDNA clones and rescue of the chimeric RABV mutants

We constructed infectious cDNA clones of six chimeric RABV mutants: rRC-HL(G), rRC-HL(NG), rRC-HL(PG), rRC-HL(NP), rRC-HL(NM) and rRC-HL(NPG). The six chimeras were generated using the genomic backbone of the RC-HL strain while the N-, P-, M- and G-encoding sequences were replaced by those of GX074. Figure 1 provides the schematic diagrams of each infectious cDNA clone.

The BSR-T7/9 cells expressing the bacteriophage T7RNA polymerase were transfected with 2.0 µg full-length plasmid and simultaneously transfected with the plasmids pIRES-N, pIRES-P or pIRES-L using Lipofectamine 2000 as previously described (Ito et al. 2001a). Transfected cells were harvested 5–7 days post-transfection. The cultures were centrifuged at 10 000 *g* at 4 °C for 10 min, and the supernatants were co-cultured with NA cells in DMEM supplemented with 2% FCS in 24-well plates for two days. IFA experiments were performed to detect the presence of the rescued RABV mutants; cells infected with the rescued virus exhibited a granular form of fluorescence of the RABV N protein (data not shown), indicating that the collected supernatant contained the rescued RABV mutants. Finally, six recombinant chimeric RABV mutants, rRC-HL(G), rRC-HL(NG), rRC-HL(PG), rRC-HL(NP), rRC-HL(NM) and rRC-HL(NPG), were obtained.

The presence of the recombinant gene in the recovered viruses was confirmed by sequencing. This analyis demonstrated that each reconstructed gene fragment was consistent with that of its parental virus (data not shown). The rRC-HL(G), rRC-HL(NG), rRC-HL(PG), rRC-HL(NP), rRC-HL(NM) and rRC-HL(NPG) mutants were successfully recovered.

### Growth properties of the rescued chimeric RABV mutants

After the chimeric RABV mutants were rescued, the N protein of each chimeric rabies virus was detected in the NA cells using IFA. The N protein was observed in the NA cells in its granular form. Furthermore, the stability of the chimeric RABV mutants was measured in the NA cells. Firstly, the chimeric RABV mutants were propagated in NA cells for 12 passages and the viral stocks were harvested after each generation and stored at –80 °C. Titration of the viral stock at the $4^{th}$, $7^{th}$ and $11^{th}$ generation (F4, F7 and F11) was carried out and the results indicated that all of the chimeric RABV mutants were stably propagated in the NA cells. The titres of parental rRC-HL were the highest (F4 = $7.1 \times 10^6$ ffu/ml, F7 = $3.7 \times 10^8$ ffu/ml, F11 = $2.8 \times 10^8$ ffu/ml; Figure 2). However, three RABV mutants, rRC-HL(NG), rRC-HL(PG) and rRC-HL(NPG), were similar to the parental virulent GX074 ($1.5 \times 10^1$, $2.5 \times 10^4$ and $3.2 \times 10^4$ ffu/ml for F4, F7 and F11, respectively), displaying stable and markedly lower

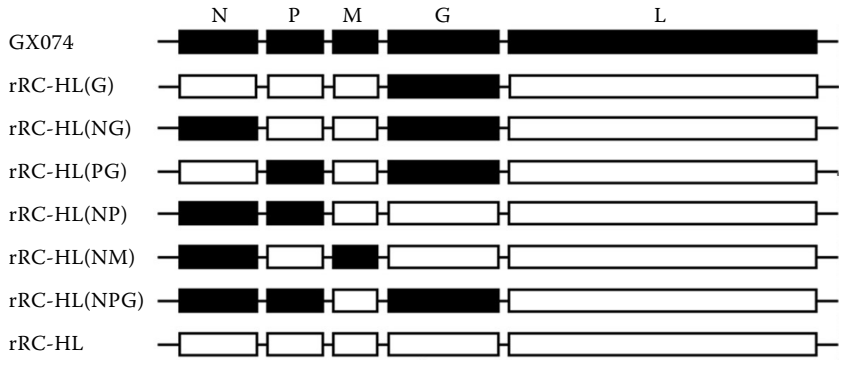


Figure 1. Construction of recombinant chimeric viral cDNA clones. Shaded and open boxes represent ORFs derived from GX074 and rRC-HL, respectively

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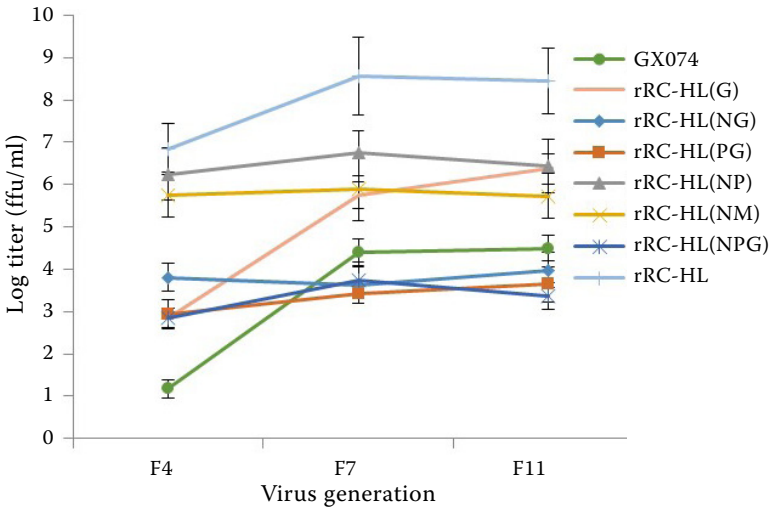


Figure 2. Titration of recombinant chimeric rabies viruses

titres than that of the avirulent parental rRC-HL. Further, the viral titres at F4, F7 and F11 of the three mutants rRC-HL(NG), rRC-HL(PG) and rRC-HL(NPG) were lower than that of rRC-HL(G). The two mutants, rRC-HL(NP) and rRC-HL(NM), were also stably propagated in NA cells, as shown by the viral titres determined at F4, F7 and F11 but the viral titres of both mutants ranged between those of the avirulent parental rRC-HL and the parental virulent GX074, including the three mutants rRC-HL(NG), rRC-HL(PG) and rRC-HL(NPG) (Figure 2).

## Pathogenicity of the chimeric RABV mutants

To assess the pathogenicity of the chimeric RABV mutants, the lethality of chimeric RABV mutant infection was determined in four-week-old Kunming mice. The mice were intracerebrally or intramuscularly injected with 30 µl of serial dilutions of RABV variants containing $10^2$ ffu or $10^6$ ffu for adult mice ($n$ = 5 per group, Table 2). The clinical symptoms were recorded 21 days post-inoculation. The body weight was measured each day. The mortality rates were evaluated after the mice were injected with serial dilutions of each strain.

## Body weight changes of mice infected with the chimeric RABV mutants

The chimeric RABV mutants rRC-HL(G), rRC-HL(NG), rRC-HL(PG), rRC-HL(NPG), rRC-HL(NP) and rRC-HL(NM) were used to intracerebrally or intramuscularly inoculate five four-week-old mice, $10^2$ ffu for the intracerebral route and $10^6$ ffu for the intramuscular route for each mouse, respectively. rRC-HL, GX074 and DMEM were used as controls. Body weight changes were measured by weighing each mouse of each group every day up until 21 dpi. The result of the intracerebral injections are shown in Figure 3A. Compared with the body weight change curve of DMEM, rRC-HL, similar to rRC-HL(NP), resulted in decreased body weight at 3 dpi which had gradually recovered at 6 dpi; the body weight change curve of rRC-HL(NM) is obviously lower than that of DMEM. The body weight change curves of avirulent parental rRC-HL and rRC-HL(NP) were intermediate between those of DMEM and rRC-HL(NM). However, DMEM, rRC-HL, rRC-HL(NP) and rRC-HL(NM) were not lethal for any mouse, although rRC-HL, rRC-HL(NP) and rRC-HL(NM) did cause minor clinical signs. GX074

Table 2. Pathogenicity of the recombinant chimeric rabies virus mutants

| Viruses | Mortality | | | |
|---|---|---|---|---|
| | $10^2$ ffu | | $10^6$ ffu | |
| | *i.c.* | *i.m.* | *i.c.* | *i.m.* |
| GX074 | 5/5 | 0/5 | 5/5 | 3/5 |
| rRC-HL(G) | 5/5 | 0/5 | 5/5 | 2/5 |
| rRC-HL(NG) | 5/5 | 0/5 | 5/5 | 1/5 |
| rRC-HL(PG) | 5/5 | 0/5 | 5/5 | 2/5 |
| rRC-HL(NP) | 0/5 | 0/5 | 0/5 | 0/5 |
| rRC-HL(NM) | 0/5 | 0/5 | 0/5 | 0/5 |
| rRC-HL(NPG) | 5/5 | 0/5 | 5/5 | 3/5 |
| rRC-HL | 0/5 | 0/5 | 0/5 | 0/5 |

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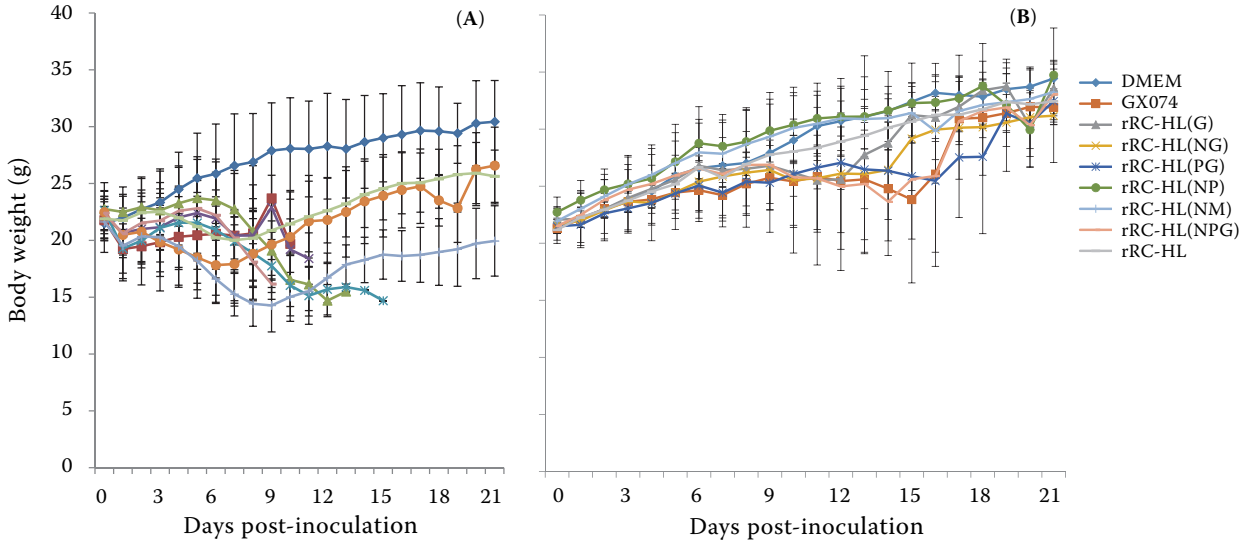


Figure 3. Body weights of mice (**A**) intracerebrally infected with chimeric rabies viruses, (**B**) intramuscularly infected with chimeric RV mutants

and the chimeric RABV mutants rRC-HL(G), rRC-HL(NG), rRC-HL(PG) and rRC-HL(NPG) not only decreased mice body weights, but were also lethal for all intracerebrally injected mice. In contrast, the body weights of mice infected with chimeric RABV mutants were markedly lower than DMEM at 3 dpi and similar to that of the parental virulent GX074. The results of intramuscular injection are shown in Figure 3B. The body weight change curves of DMEM, rRC-HL, rRC-HL(NP) and rRC-HL(NM) exhibited no reductions after the injection. GX074 and the chimeric RABV mutants rRC-HL(G), rRC-HL(NG), rRC-HL(PG) and rRC-HL(NPG) resulted in decreased body weights, and those mice became ill and eventually died. Body weight curves of rRC-HL(NPG) and rRC-HL(NG) were closer to that of GX074, but lower than rRC-HL(G), suggesting that the N, P and G genes of GX074 can collaborate to promote RABV pathogenicity.

## Lethality of the chimeric RABV mutants

The results demonstrated that the mice intracerebrally inoculated with $10^2$ ffu or $10^6$ ffu of the rRC-HL, rRC-HL(NP) and rRC-HL(NM) strains developed mild disease with symptoms such as piloerection and body weight reduction, but no obvious neurological symptoms, and none of them died. In contrast, all five mice that were intracerebrally injected with $10^2$ ffu or 1 : 5 mice brain emulsions of GX074 died of rabies and presented with the classical neurological symptoms, including sensitivity to stimuli, remaining largely immobile in the corner of the cage and depression at 7 dpi; excitement, apparent emaciation, mania and random running at 8 dpi; and general tremors, hind limb paralysis, respiratory failure and death at 9 dpi (Tables 2 and 3). Similar to what was observed for the GX074 treatment, all of the mice intracerebrally inoculated with $10^2$ ffu or 1 : 5 mice brain emulsions of the rRC-HL(NG), rRC-HL(PG) or rRC-HL(NPG) mutant died after exhibiting severe neurological symptoms (Tables 2 and 3). However, in the case of intramuscular injection, all the mice inoculated with $10^2$ ffu of any of the RABV mutants, including GX074, survived. For the mice receiving intramuscular injections of 1 : 5 mice brain emulsions, 20% (1/5) of those receiving the rRC-HL(NG) variant and 40% (2/5) of those receiving the rRC-HL(PG) variant died (in both cases, obvious neurological symptoms were observed), while treatment with the rRC-HL(NPG) variant resulted in lethality for 60% (3/5) of mice, the same as when the virulent parental GX074 when injected intramuscularly. These data suggest that the N, P and G gene cooperate in the pathogenicity of the RABV. As presented in Table 3, the mice inoculated with the chimeric RABV mutants developed different symptoms, including typical RABV neurological symptoms such as paralysis and fury.

Duration of survival after intracerebral and intramuscular infection routes is shown in Figure 4A and Figure 4B. Mice died 8–16 days after intracerebral administration of $10^2$ ffu rRC-HL(G), rRC-HL(NG),

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Table 3. Clinical symptoms in mice infected with the recombinant chimeric viruses

| | Viruses | Days post-inoculation (dpi) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12–21* |
| Intracerebral injection site | GX074 | – | – | – | ± | + | ++ | ++ | +++ | ++++ | – | | |
| | rRC-HL(G) | – | – | – | – | – | ± | + | ++ | +++ | ++++ | | |
| | rRC-HL(NG) | – | – | – | – | ± | + | ++ | +++ | ++++ | – | | |
| | rRC-HL(PG) | – | – | – | – | – | + | +++ | +++ | ++++ | – | | |
| | rRC-HL(NP) | – | – | – | – | + | + | + | – | – | – | | |
| | rRC-HL(NM) | – | – | – | – | + | ++ | ++ | ± | – | – | | |
| | rRC-HL(NPG) | – | – | – | – | ± | + | ++ | +++ | ++++ | – | | |
| | rRC-HL | – | – | – | + | + | + | + | – | – | – | | |
| Muscle injection site | GX074 | – | – | – | – | – | – | – | ++ | +++ | ++++ | ++++ | ++++ |
| | rRC-HL(G) | – | – | – | – | – | – | – | – | ± | + | ++ | ++++ |
| | rRC-HL(NG) | – | – | – | – | – | – | – | ++ | +++ | ++++ | ++++ | ++++ |
| | rRC-HL(PG) | – | – | – | – | – | – | – | ++ | +++ | ++++ | ++++ | ++++ |
| | rRC-HL(NP) | – | – | – | – | – | – | – | – | – | – | – | – |
| | rRC-HL(NM) | – | – | – | – | – | – | – | – | – | – | – | – |
| | rRC-HL(NPG) | – | – | – | – | – | – | + | ++ | ++++ | ++++ | ++++ | ++++ |
| | rRC-HL | – | – | – | – | – | – | – | – | – | – | – | – |

*Partial mice died between the 12th and 21st day, presented ++++ clinical signs

– = normal, ± = dubious and asymptomatic, + = disordered fur, inappetence, slow movement and hypokinesia, ++ = stimuli-sensitive, remaining largely immobile in the corner of the cage and depression, +++ = excitement, apparent emaciation, mania and random running, ++++ = general tremors, hind limb paralysis, respiratory failure and death

rRC-HL(PG) and rRC-HL(NPG) and 13–19 days after $10^6$ ffu inoculated through the intramuscular route. Mice intracerebrally infected with rRC-HL showed slight clinical symptoms at 4–7 days, but almost no clinical signs could be seen in the mice injected in muscle. The mice injected with the parental GX074 began to fall sick at 4 dpi, displayed typical RABV neurological signs from 7 dpi and begin to die at 8 dpi. Among the mutants, rRC-HL(NP) and rRC-HL(NM) were similar to rRC-HL, only showing slight clinical symptoms at 5–8 days followed by recovery; however, no clinical signs could be seen in the mice injected in muscle. The mice injected with the three mutants RC-HLΔNG,

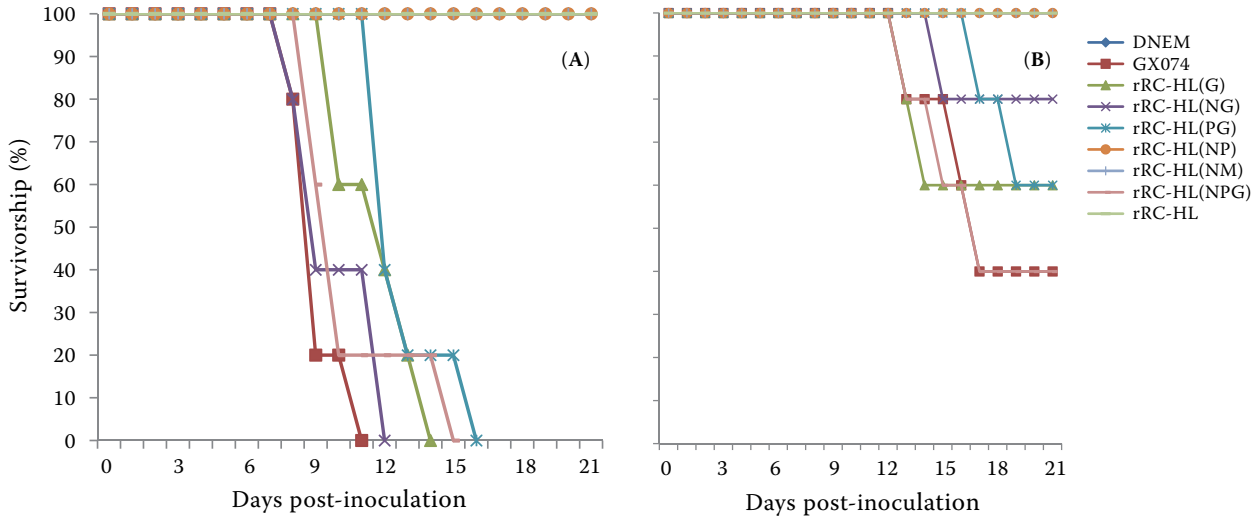


Figure 4. Survival of mice (**A**) intracerebrally infected with chimeric RV mutants, (**B**) intramuscularly infected with chimeric RV mutants

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rRC-HL(PG) and rRC-HL(NPG) showed clinical symptoms that were similar to mice receiving intracerebral or muscle injection. However, the mice injected with mutant rRC-HL(G) intracerebrally began to display symptoms at 6 dpi and those injected in muscle by 9 dpi; the symptoms were markedly less pronounced than those elicited by the three mutants RC-HLΔNG, rRC-HL(PG), rRC-HL(NPG) and parental GX074 (Table 3).

### $LD_{50}$ of the chimeric RABV mutants

To further assess the pathogenicity of the chimeric RABV mutants, the $LD_{50}$ values of the chimeric RABV mutants rRC-HL(G), rRC-HL(NG), rRC-HL(PG) and rRC-HL(NPG) were tested. Compared to the $10^{-7.19}$ of parental GX074, the chimeric RABV mutants rRC-HL(G), rRC-HL(NG), rRC-HL(PG) and rRC-HL(NPG) displayed $LD_{50}$ values of $10^{-5.35}$, $10^{-5.84}$, $10^{-6.02}$ and $10^{-6.35}$, respectively. The $LD_{50}$ of rRC-HL(G) was higher than that of rRC-HL(NG), rRC-HL(PG), rRC-HL(NPG) and parental GX074 ($10^{-7.19}$) (Table 4). On the other hand, the clinical signs of mice inoculated with chimeric RABV mutants were basically consistent with their $LD_{50}$ values (data not shown). Specifically, the course of sickness for rRC-HL(G) lasted an average of 4.07 days, while the courses of sickness for rRC-HL(NG), rRC-HL(PG), rRC-HL(NPG) and parental GX074 were 2.50, 3.33, 3.08 and 1.50 days, respectively (Table 4). These findings also indicated that the N and P genes are involved in and facilitate the pathogenicity of the rabies virus G gene.

## DISCUSSION

Previous studies have demonstrated that the G protein is important for the neuroinvasiveness of the RABV (Prehaud et al. 1988; Morimoto et al. 2000; Mazarakis et al. 2001; Faber et al. 2004; Mentis et al. 2006; Pulmanausahakul et al. 2008; Virojanapirom et al. 2012; Yamada et al. 2012), and, specifically, that the arginine or lysine residue at position 333 is required for lethality in adult mice. This phenomenon is common among representative laboratory strains of the rabies virus, including the HEP-flury, CVS and SAD B19 strains (Dietzschold et al. 1983; Seif et al. 1985; Diallo 1986; Tuffereau et al. 1989; Mebatsion 2001). It has been reported that pathogenic RABV suppresses its replication rate to maintain its pathogenicity (Dietzschold et al. 2004; Pulmanausahakul et al. 2008). In this study, we also found that the G protein is the key virulent protein in the street virus isolate GX074. The viral titre of GX074 and the recombinant chimeric rRC-HLΔG was lower than that of the parental rRC-HL in NA cells. This indicates that the low replication efficiency of the rabies virus is determined by the G protein, which is consistent with the previous observation that replacement of the B2c G gene with the G gene from street RABVs (SHBRV and DRV) resulted in decreased viral replication (Zhang et al. 2013).

Table 4. Pathogenicity of the recombinant chimeric rabies virus mutants

| Virus | Course of sickness (days) | $LD_{50}$ |
|---|---|---|
| GX074 | 1.50 | −7.19 |
| rRC-HLΔG | 4.07 | −5.35 |
| rRC-HLΔNG | 2.50 | −5.84 |
| rRC-HLΔPG | 3.33 | −6.02 |
| rRC-HLΔNPG | 3.08 | −6.35 |
| rRC-HL | 3.50 | > 5.34 |

The idea that viral pathogenicity is controlled by multigenic factors has been suggested for the influenza virus (Hatta et al. 2001), Newcastle disease virus (Huang et al. 2004) and Rinderpest virus (Baron et al. 2005) based on reverse genetics approaches. Therefore, we attempt to examine whether in addition to the G gene, the other RABV genes N, P and M are also involved in its pathogenicity. Six chimeric RABV mutants, rRC-HL(NP), rRC-HL(NM), rRC-HL(G), rRC-HL(NG), rRC-HL(PG) and rRC-HL(NPG), were generated. On the basis of their growth properties, the viral titres of the two mutants rRC-HL(NP) and rRC-HL(NM) were intermediate between that of the parental rRC-HL and GX074 levels. The viral titre of mutant rRC-HL(G) was lower than that of mutants rRC-HL, rRC-HL(NP) and rRC-HL(NM), but higher than that of rRC-HL(NG), rRC-HL(PG), rRC-HL(NPG) and the parental GX074 strain.

Both mutant rRC-HL(NP) and rRC-HL(NM) were not lethal for four-week-old mice. All the mice inoculated with $10^2$ ffu and $10^6$ ffu of RABV mutants rRC-HL(G), rRC-HL(NG), rRC-HL(PG), rRC-HL(NPG) and parental GX074 died (5/5, 100%). In the case of intramuscular viral infection, all the mice inoculated with $10^2$ ffu of any of the RABV mutants, including GX074, survived; at

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$10^6$ ffu, rRC-HL(NG) was lethal for one of five and rRC-HL(PG) was lethal for two of five mice that exhibited characteristic neurological symptoms. However, the rRC-HL(G) mutant was lethal for two of five mice when administered intramuscularly. There was a positive correlation between the body weight loss rate (Figure 3) of mice and the severity of clinical symptoms (Table 3) and the pathogenicity. Furthermore, the $LD_{50}$ values of the chimeric RABV mutants were measured, with the results showing that the $LD_{50}$ values of the two mutants rRC-HL(NG) and rRC-HL(PG) were lower than that of rRC-HL(G), but higher than that of rRC-HL(NPG). Taken together, this demonstrates that the pathogenicity of the combined action of RABV N + G, P + G, or N + P + G genes might be stronger than that of the G gene alone, indicating that the N, P and G genes collaborate in governing the pathogenicity of the rabies virus. The probable reason for this phenomenon is that the nucleoprotein of virulent virus strains functions to evade activation of the RIG-I-mediated antiviral response (Masatani et al. 2010); P protein interferes with phosphorylation of IRF3 (Brzozka et al. 2005), interacts with STAT1 and inhibits interferon signal transduction pathways (Vidy et al. 2005), mechanisms that together allow the virus to evade induction of host IFN and chemokines and which correlate with viral pathogenicity (Ito et al. 2010).

In addition, we also constructed infectious cDNA clones of rRC-HLΔMG, rRC-HLΔPMG and rRC-HL(NPMG) but were unable to recover these chimeric RABV mutants despite exhaustive efforts. The reason for this failure is unknown. The interaction between the G protein and the RNP-M complex is crucial for efficient replication and budding of the viral particles (Mebatsion et al. 1999). Further research on the pathogenicity of RABV using the mutant rRC-HL(G) is ongoing.

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