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BRIEF REPORT

Adaptation of a Chinese ferret badger strain of rabies virus to high-titered growth in BHK-21 cells for canine vaccine development

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Abstract Rabies virus strain JX08-45CC was derived from a Chinese isolate (JX08-45) by serial passage in the BHK-21 cell line, reaching a titer of 10^8 $TCID_{50}$/mL. JX08-45CC produced rabies in adult mice but was non-pathogenic in dogs after intramuscular injection. A comparison of the entire genomes of JX08-45 and JX08-45CC led to the identification of 17 nucleotide substitutions, resulting in seven amino acid changes in the mature G and L proteins. The immunogenicity of β-propiolactone-inactivated JX08-45CC was similar to the immunogenicity of the live vaccine strains widely used in China. The inactivated vaccine induced antibody responses for more than 6 months and provided full protection from an intramuscular challenge in dogs. JX08-45CC has excellent potential for development as an inactivated vaccine for dogs in China.

Keywords rabies virus · Chinese ferret badger · cell-culture-adapted virus · vaccine

In China, the coverage of rabies immunization in dogs is low. Thus, more than 95 % of the 2000 to 3000 human rabies cases reported annually are caused by rabid dog bites [1, 2]. All Chinese lyssavirus isolates are genotype I and provide full cross-protection, indicating that any rabies vaccine strain of local origin offers countrywide protection against naturally occurring rabies viruses [3]. The use of live, attenuated vaccines is no longer recommended for parenteral immunization in animals by the World Health Organization (WHO) Expert Consultation on Rabies (2004; Geneva). Inactivated vaccines are the most commonly used anti-rabies biologics for domestic animals. However, no locally produced veterinary inactivated rabies vaccine is available in the Chinese market [4]. In China, domestic live vaccines (including ERA and Flury-LEP) that are weakly potent in dogs and have poor safety records in young animals are still widely used [4, 5]. Considering their low titers in cell cultures resulting from the genetic drift of strains and/or disturbance of production technology in China, these live vaccine strains require expensive concentration processing to improve their potency, thereby prohibiting their use in the local production of inactivated vaccines [4, 5]. In this study, we describe a highly cell-culture-adapted rabies virus derived from a Chinese street isolate. Given the high immunogenicity and good growth characteristics of the virus, it has excellent potential for development as a veterinary inactivated vaccine.

Brain tissue samples of Chinese ferret badgers (*Melogale moschata*) were collected in Jiangxi Province, China, in December 2008. A rabies street virus was isolated from one animal (#45) by intracerebral (i.c.) injection of a 1-day-old suckling mouse, and the strain was named JX08-45. This strain was closely related to known Chinese dog rabies virus lineages according to a previous phylogenetic analysis [6]. Rabies virus strains ERA, SRV_9, and Flury-LEP, all widely used in live veterinary vaccine preparations in China, were cultivated in baby hamster kidney (BHK)-21 cells in Dulbecco's minimum essential medium (DMEM) supplemented with 2 % newborn calf serum, 100 U/mL penicillin G, and 100 μg/mL streptomycin sulfate at 37 °C in a 5 % CO_2 humidified incubator. For mouse vaccination studies, following titer adjustment to $10^{6.0}$ $TCID_{50}$/mL, the strains were inactivated using 1/4000

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β-propiolactone (BPL; Wako Pure Chemical Industries, Ltd., Japan). Viral inactivation was verified using WHO-recommended tests for effective inactivation [7]. A live CVS-11 virus was used for the fluorescent antibody virus neutralization (FAVN) test to measure neutralizing antibody titers [8]. Rabies virus strain BD06 (GenBank #EU549783.1), isolated in our laboratory in 2006 from a rabid Chinese dog, was maintained in dog brain and propagated once in adult mouse (14–16 g) brain for challenge. Passage in mouse brain was limited to prevent the reduction of its pathogenicity in dogs. A virus suspension was prepared by low-speed centrifugation from 10 % mouse brain homogenates in PBS (pH 7.4) containing 2 % horse serum and stored at −80 °C. The titer was $10^{4.5}$ mouse i.c. LD_{50}/0.03 mL. For the rabies virus challenge of dogs via the masseter muscle, the stock of BD06 was diluted to 6×10^4 mouse i.c. LD_{50}/0.03 ml injection in PBS/2 % horse serum. Adult Kunming mice (14–16 g) were purchased from the Changchun Institute of Biological Products. Unvaccinated beagles (5–7 kg, 3–4 months old) were purchased from Beijing Marshall Biotechnology Co., Ltd. All animals were treated humanely and euthanized by cervical dislocation (mice) or intravenous injection of 5 % pentobarbital (dogs) at the end of the experimental period.

A suckling mouse brain tissue suspension of JX08-45 (first mouse passage) was clarified by centrifugation at $5000 \times g$ for 5 min, and 0.2 ml of the supernate was added to a BHK-21 cell monolayer in a 25-cm^2 cell culture flask (EasyFlasks™, Thermo Fisher Scientific, Denmark). After 1 h at 37 °C to permit virus adsorption, the cell monolayer was rinsed with PBS (pH 7.4) and incubated in DMEM/2 % newborn calf serum at 34 °C in a 5 % CO_2 humidified incubator. The infected cells were serially passaged every 8 days at 34 °C, with samples being taken every 10 passages for virus titration following three freeze-thaw cycles. This step continued until an acceptable virus titer for vaccine production was reached ($\geq 10^8$ $TCID_{50}$/ml). The resulting cell-culture-adapted JX08-45 was renamed JX08-45CC (GenBank JQ946087). ERA and Flury-LEP were cultured with JX08-45CC as the control. A $TCID_{50}$ assay of the harvested virus was performed by direct immunofluorescence [9]. Monolayers of BHK-21 cells in 96-well plates (8 wells/dilution) were incubated with serial tenfold virus dilutions. At 48 h postinfection, the cells were fixed in 80 % acetone and stained with FITC-labeled rabies virus N-protein-specific monoclonal antibody (made in our laboratory). The plates were examined by fluorescence microscopy (Olympus Corp., Tokyo, Japan), and the presence or absence of fluorescent foci in the cells was recorded. Endpoints were defined as the highest dilutions showing fluorescent foci, and virus titers ($TCID_{50}$) were calculated by the Spearman–Kärber formula.

Serial tenfold dilutions of JX08-45CC (120^{th} passage in BHK-21 cells) were prepared in PBS/2 % horse serum and injected i.c. into seven groups of 10 adult mice (0.03 mL, containing $0.3–3 \times 10^5$ $TCID_{50}$; one dilution per group). Thereafter, five groups of 10 adult mice were injected i.m. with 0.1 mL (containing $10^3–10^7$ $TCID_{50}$) in the hindlimb skeletal muscle. Negative controls received only PBS. To compare the pathogenicities of JX08-45 and JX08-45CC at the same dose in mice, the two strains were cultivated in a mouse neuronal-2a (N2a) cell line in RPMI1640 medium supplemented with 10 % fetal bovine serum, 100 U/mL penicillin G, and 100 μg/mL streptomycin sulfate at 37 °C in a 5 % CO_2 humidified incubator. Only two passages in N2a cells were performed to prevent pathogenicity reduction, and the titers of JX08-45 and JX08-45CC were $10^{3.0}$ and $10^{3.75}$ $TCID_{50}$/mL, respectively. Serial tenfold dilutions of JX08-45 and JX08-45CC (second passage in N2a cells) were prepared in PBS/2 % horse serum and injected i.c. into three groups of 10 adult mice (0.03 mL, containing 0.3–30 $TCID_{50}$; 1 dilution per group). Another three groups of 10 adult mice were injected i.m. with 0.1 mL (containing $1–10^2$ $TCID_{50}$) in the hindlimb skeletal muscle. The numbers of mice dying of rabies 5 and 28 days after challenge were recorded, and rabies virus infection in their brain tissue was confirmed by the direct fluorescent antibody test (FAT) [9]. Twenty unvaccinated beagle dogs (10/group) were injected i.m. (right masseter muscle) with 0.5 mL of JX08-45CC or JX08-45 (containing 10^5 mouse i.c. LD_{50} each) and then observed daily for clinical signs of rabies for 90 days post-challenge. Negative controls (10 dogs) received only PBS. Sections of Ammon's horn and cerebellar tissues were examined post-mortem for rabies by the direct FAT. A serum sample was collected from each surviving animal for FAVN test at the end of the experimental period.

Genomic RNA was extracted from JX08-45CC at passage levels 40, 80 and 120 using TRIzol Reagent (Invitrogen Life Technologies, USA) according to the manufacturer's instructions. The full-length cDNA was synthesized using reverse primers based on the wild-type JX08-45 genome sequence (GenBank no. GU647092.1). The viral genome was amplified as nine overlapping PCR fragments covering the full length of its cDNA. The 3′- and 5′-terminal fragments of the JX08-45CC genome were obtained using a 3' and 5' Full RACE Kit (TaKaRa Biotechnology Co., Ltd., Dalian, China). The PCR products were purified, and nucleotide sequencing was performed on both the forward and reverse strands of each fragment by Genscript Nanjing Co., China. The nucleotide sequences of the complete genomes of JX08-45 and JX08-45CC were compared with those of some other vaccine and isolate strains: CTN-1 (GenBank FJ959397.1), ERA (GenBank EF206707.1), SAG_2 (GenBank EF206719.1), SRV_9

(GenBank AF499686.2), BD06 (GenBank EU549783.1), 8743THA (GenBank EU293121.1), NNV-RAB-H (GenBank EF437215.1), and DRV-Mexico (GenBank HQ450 386.1) using DNASTAR Lasergene® software (DNASTAR, Inc., Madison, USA).

Adult mice were randomized, and four groups of 10 animals were injected i.m. in the left quadriceps with JX08-45CC, ERA, SRV9, or Flury-LEP (0.05 mL of inactivated virus, containing 5×10^5 $TCID_{50}$). Negative controls (10 animals) received only PBS. Serum samples were collected from the retro-orbital plexus 14 days after treatment. About 90 mL of inactivated virus JX08-45CC (originally 10^8 $TCID_{50}$/mL) mixed with 10 mL of aluminum hydroxide gel (13 mg/mL, batch no. MKBD7814, Sigma-Aldrich, USA) was used as dog rabies vaccine. Nobivac® Rabies (batch bo. A154A01, Intervet International) was selected as a positive control. For the immunogenicity studies of JX08-45CC as a vaccine candidate for dogs, 30 dogs were randomly divided into three groups. Two groups were injected with 1 mL of JX08-45CC or Nobivac® Rabies in the triceps brachii muscle. The third group (negative control) received only PBS. Serum samples were collected from the brachial vein before vaccination and every month thereafter for 6 months. About 6 months post-vaccination, the dogs were challenged i.m. (masseter muscle) with BD06 virus (6.0×10^4 mouse LD_{50}) and observed for 90 days for signs of rabies. All animals exhibiting clinical symptoms were euthanized with 5 % barbiturate solution administered intravenously, and brain impressions were made to test for rabies virus antigen by FAT [9].

Variance analysis was performed using SPSS 16.0 software for Windows (SPSS Inc., Chicago, IL, USA) to determine statistically significant differences in the generated data by one-way ANOVA. The results of the comparisons between groups were considered significantly different if $p < 0.05$, and very significantly different if $p < 0.01$. Data are expressed as mean ± SD.

With serial passage in BHK-21 cells, the titer of JX08-45 virus gradually increased, reaching a plateau at passage level 120 that remained at about $10^{8.0}$ $TCID_{50}$/mL. The titers of the virus increased rapidly initially, reaching $10^{4.25}$ $TCID_{50}$/mL by the 40th passage and $10^{7.0}$ $TCID_{50}$/mL by the 80th passage. However, further passage increased the titer by only 10-fold, with no increase between passages 120 and 130. The titers of ERA and Flury-LEP only reached $10^{6.0}$ and $10^{6.25}$ $TCID_{50}$/mL.

Table 1 shows that all adult mice died after i.c. JX08-45CC injection at doses $>3\times10^4$ $TCID_{50}$. At lower titers, some animals survived. Following i.m. injection, some animals survived even doses as high as 3×10^6 $TCID_{50}$, developing rabies-neutralizing antibody titers ranging from 1.14–53.3 IU/mL. None of the mice died after i.c.

Table 1 Pathogenicity of JX08-45 and JX08-45CC in mice

Virus	Cell type	Challenge route	Deaths per 10 animals									
			Viral dose (i.c. /i.m., $TCID_{50}$)	0/0	0.3/1	$3/10^1$	$30/10^2$	$3 \times 10^2/10^3$	$3 \times 10^3/10^4$	$3 \times 10^4/10^5$	$3 \times 10^5/10^6$	$3 \times 10^6/10^7$
JX08-45CC	BHK-21	i.c.		0	0	2	3	5	7	8	10	—
		i.m.		0	—	—	—	0	1	1	4	7
	N2a	i.c.		0	0	2	3	—	—	—	—	—
		i.m.		0	0	0	0	—	—	—	—	—
JX08-45	N2a	i.c.		—	1	4	10	—	—	—	—	—
		i.m.		—	0	1	3	—	—	—	—	—

"—": not done

JX08-45CC injection at doses <3 $TCID_{50}$ in N2a cells and i.m. JX08-45CC injection at doses $<3\times10^3$ $TCID_{50}$ in BHK-21 cells. However, all adult mice died after i.c. JX08-45 injection at doses >3 $TCID_{50}$ in N2a cells. Following i.m. injection, some animals survived even doses as high as 30 $TCID_{50}$/mL in N2a cells. When present, rabies symptoms were observed 7–9 days post-challenge, with deaths occurring within the following 24–48 h. All negative control mice remained alive and healthy. About 90 d post-challenge, none of the dogs injected with JX08-45CC developed rabies and died; however, three dogs given JX08-45 showed symptoms such as foaming at the mouth, paralysis of the chewing muscles and hind legs, and confusion at 11, 16, and 17 days respectively, with death occurring within 2–3 days. These data indicate that JX08-45CC is nonpathogenic in dogs at an i.m. dose of 10^5 mouse i.c. LD_{50} and induces a strong protective immune response. The titers of rabies-neutralizing antibody 90 days after inoculation ranged from 2.60 to 23.4 IU/mL. All negative control dogs remained alive and healthy.

As shown in Table 2a, the genomes of JX08-45 and JX08-45CC each contain 11,922 nt, with 17 nt changes in the cell-culture-adapted virus. One nucleotide substitution was located in the untranslated region between the P and M coding regions, with seven substitutions in the G coding region and eight in the L coding region. No changes were observed in any other part of the viral genome. The nucleotide substitutions resulted seven amino acid changes (genome [nt] positions 3523, 3761, 3925, 4110, 4414, 4646, and 5484), with one in the L protein (nt 5484) and the other six located in the mature G protein at amino acid positions 51, 130, 191, 247, 348, and 425. A comparison of the genome sequence of the JX08-45CC strain with less-passaged strains revealed that only one amino acid (Pro, genome [nt] position 3761) was changed to Ser in 40 passages in BHK-21, and four amino

Table 2a Comparison of genome sequences of JX08-45CC strains with those of less-passaged strains

Genome location (nt)	Protein position*	JX08-45		40 passages in BHK-21		80 passages in BHK-21		JX08-45CC	
		Nucleotide	Amino acid	Nucleotide	Amino acid	Nucleotide	Amino acid	Nucleotide	Amino acid
2469	UTR	A	—	A	—	G	—	G	—
3523	G-51	T	Ile	T	Ile	G	Ser	G	Ser
3761	G-130	C	Pro	T	Ser	T	Ser	T	Ser
3925	G-191	C	Ser	C	Ser	T	Leu	T	Leu
4110	G-247	G	Asp	G	Asp	A	Asn	A	Asn
4414	G-348	G	Gly	G	Gly	G	Gly	A	Glu
4418	G	C	—	A	—	A	—	A	—
4646	G-427	G	Lys	G	Lys	G	Lys	T	Asn
5484	L-26	A	Thr	A	Thr	C	Pro	C	Pro
5576	L	A	—	A	—	G	—	G	—
6881	L	T	—	G	—	C	—	C	—
7287	L	G	—	A	—	A	—	A	—
8161	L	C	—	C	—	T	—	T	—
10154	L	G	—	G	—	G	—	A	—
10188	L	C	—	A	—	A	—	A	—
11231	L	T	—	T	—	C	—	C	—

" " represents changed nucleotides from JX08-45 "—" represents unchanged amino acids from JX08-45 "□" represents changed amino acids from JX08-45

"*" represents the position of changed amino acids in the mature protein

acid substitutions (genome [nt] positions 3523, 3925, 4110, and 5484) were found in the process from 40 to 80 passages in BHK-21.

Amino acid comparisons with other rabies vaccine strains revealed that four of the seven substitutions from JX08-45 (genome [nt] positions 3761, 3925, 4110, and 4646) were generally similar to those found at the same genome positions in the other vaccine strains examined (Table 2b). However, only two of the four substitutions (genome [nt] positions 4110 and 4646) were not found at the same genome positions in the street rabies virus isolates examined.

In the comparison of the immunogenicity of three vaccine strains and JX08-45CC, antibody titers were analyzed by one-way ANOVA, setting $F = 0.649$, $P = 0.63 > 0.05$; i.e., the differences in the mean values were not statistically significant. Numerically, the mean JX08-45CC titer (7.38 ± 5.58 IU/mL) was almost equal to that of Flury-LEP (7.66 ± 4.90 IU/mL) and slightly higher than those of ERA (4.64 ± 4.53 IU/mL) and SRV_9 (6.83 ± 5.68 IU/mL). The specific antibody was not detected in the negative controls. As summarized in Fig. 1, the immunological efficacy of the JX08-45CC and Nobivac® Rabies products was monitored each month after vaccination. The antibody titers stimulated by both vaccines reached a peak in the first month, and both gradually declined by 50 % by 6 months post-vaccination. Ninety days post-challenge, all dogs injected with either vaccine remained normal, whereas all negative-control dogs developed rabies between 8 and 24 days post-inoculation.

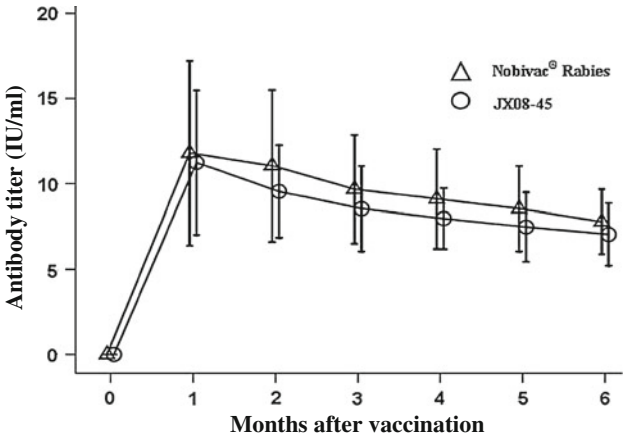

Fig. 1 Rabies-neutralizing antibody responses in dogs after immunization with Nobivac Rabies and JX08-45CC. Error bars: mean ± S.D

The passage of a virus *in vitro* generally reduces its ability to replicate in its natural host, resulting in a reduction in the pathogenicity of the latter [10–12]. JX08-45CC retained a certain degree of pathogenicity in adult mice, but only at high titers. In dogs, JX08-45CC was nonpathogenic at the highest tested dose and induced a strong protective immune response, whereas three out of 10 dogs injected i.m. with the parent JX08-45 died of rabies. Glycoprotein is the most relevant component of the rabies virus because of its pathogenicity and some other functions such as attachment to host cells [13–15]. Therefore, the clustering of JX08-45CC in this viral protein is expected. Previous studies have implicated the presence of Arg-333

Table 2b Comparison of the genome sequence of the JX08-45CC strain with those of other vaccine and isolate strains

Genome location (nt)	Amino acid substitutions (JX08-45→JX08-45CC)	Amino acid at the same genome location of other vaccine strains				Amino acid at the same genome location of other isolates			
		CTN-1	SAG_2	SRV_9	ERA	BD06	8743THA	NNV-RAB-H	DRV-Mexico
3523	Ile→Ser	Ile	Ile	Ile	Ile	Ser	Ser	Ile	Ile
3761	Pro→Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser
3925	Ser→Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu
4110	Asp→Asn	Asn	Asn	Asn	Asn	Asp	Asp	Asp	Asp
4414	Gly→Glu	Gly	Gly	Gly	Gly	Gly	Gly	Gly	Gly
4646	Lys→Asn	Lys	Asn	Asn	Asn	Lys	Glu	Lys	Lys
5484	Thr→Pro	Thr	Ile	Ile	Thr	Thr	Thr	Thr	Thr

"□" represents changed amino acids from JX08-45 (genome [nt] positions 4110 and 4646). These amino acids were found at the same genome positions in the other vaccine strains examined but not at the same genome positions in the other street strains examined

and Asn-194 in mature glycoproteins in rabies viral pathogenicity or the re-emergence of the pathogenic phenotype of a rabies virus strain [16, 17]. Likewise, amino acids at positions 242, 255, and 268 have been found to be associated with the pathogenicity of rabies virus strains in adult mice [18]. However, none of these virulence-associated amino acids were changed in the conversion of virulent JX08-45 into attenuated JX08-45CC. Little is known about the changes occurring in G and L proteins during the cell culture adaptation of rabies virus [19]. In the present study, none of the seven amino acid substitutions observed in JX08-45CC were located in any of the above pathogenicity-associated positions, or even in any of the three major antigenic sites [20–25]. Following passage in BHK-21 cells, these seven amino acids were gradually changed, and four of the amino acid substitutions reverted to the same ones found at the corresponding genome positions in most other cell-culture-adapted strains examined. Two out of the four amino acid substitutions were not found at the same genome positions in the other street rabies virus isolates examined. This finding suggests that some, if not all, of these changes are involved in viral attenuation and adaptation to cell culture. In the mouse immunization test, the immunogenicity of JX08-45CC was similar to the best of the live vaccine strains widely used in China in the BPL-inactivated state. The inactivated vaccine based on JX08-45CC can provide full protection from challenge with a Chinese street virus in dogs. Our data indicate that the immunogenicity of JX08-45CC is sufficient to permit inactivated vaccine production without the need for further concentration.

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Conflict of interest The authors declare that they have no conflict of interest.

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