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LETTER

A Highly Attenuated Mumps Virus Strain of Genotype F Generated by Passaging in Vero Cells

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Dear Editor

Mumps, caused by mumps virus (MuV), is still a serious threat to the health of children and teenagers. In most mumps cases, the clinical symptoms were parotitis, myalgia, malaise, fever and headache. As a neurotropic virus, MuV infection also causes cerebrospinal fluid pleocytosis in one-third of patients and meningitis in 10% of patients. For very few cases, orchitis and oophoritis also can occur (Carbone and Wolinsky 2001).

MuV, a member of genus *Rubulavirus* within the family *Paramyxoviridae*, contains a single-stranded negative sense RNA which composed of 15384 nucleotides (Rubin *et al.* 1998). The genome of MuV encodes six structural proteins (nucleoprotein, phosphoprotein, matrix protein, fusion protein, hemagglutinin-neuraminidase protein, large protein) and three non-structural proteins (V protein, I protein and SH protein). Although MuV has only one serum type, it could be divided into 12 genotypes (A–N, excluding E and M) (WHO 2012) according to the sequence of *SH* gene which are distinct from each other (Turner *et al.* 1991).

Currently, the mumps vaccine strains used in most of countries in the world all belong to genotype A, which is no longer an epidemic genotype. Genotype F is the predominant MuV genotype in China's mainland. Reportedly, 97.33% of 451 strains of MuV isolated in China from 2001 to 2017 were conformed as genotype F (Cui *et al.* 2014; Cui *et al.* 2017; Cui *et al.* 2018). Although the Measles-mumps-and-rubella vaccine (MMR) has been introduced in the Expanded Programme on Immunization in 2008, there have been local outbreaks of disease in vaccinated populations every year in China (Zengel *et al.* 2017). More and more studies are confirming that it is necessary to develop novel genotype mumps vaccine. (Dayan and Rubin 2008; Carr *et al.* 2010; Park 2015).

In this study, 29 strains of MuV have been isolated from laryngeal swab samples collected from sick children in 2016 in six provinces of China (Jiangsu, Zhejiang, Hubei, Shanxi, Guangdong and Beijing) (Supplementary Table S1), and all the *SH* genes were sequenced. The phylogenetic analysis showed that all 29 MuVs belong to Genotype F (Fig. 1A) including two strains isolated from Zhejiang Province, 11 from Jiangsu Province, one from Hubei Province, one from Beijing, six from Shanxi Province, and eight from Guangdong Province. A strain isolated from Jiangsu Province, named as QS-F (preservation No. CCTCC No.V201948), exhibited good replication capacity in Vero cells (Fig. 1B). The MuV strain QS-F was purified by a standard plaque purification technique and screened for three times with the biggest plaque selected each time (see supplementary Methods). Then three time-purified QS-F was continuously passaged in Vero cells to the 30th passage. The viral titers of QS-F at 3rd, 7th, 10th, 13th, 16th, 20th, 25th, and 30th passage were 6.25 ± 0.35, 5.875 ± 0.53, 6.0, 6.37 ± 0.17, 7.5, 7.375 ± 0.17, 7.0 and 7.25 ± 0.35 $lgCCID_{50}$/mL, respectively (Fig. 1C). Obviously, the proliferative ability of QS-F in Vero cells was improved with the passaging (Fig. 1C). To evaluate the replication kinetics of QS-F after passaging for three times

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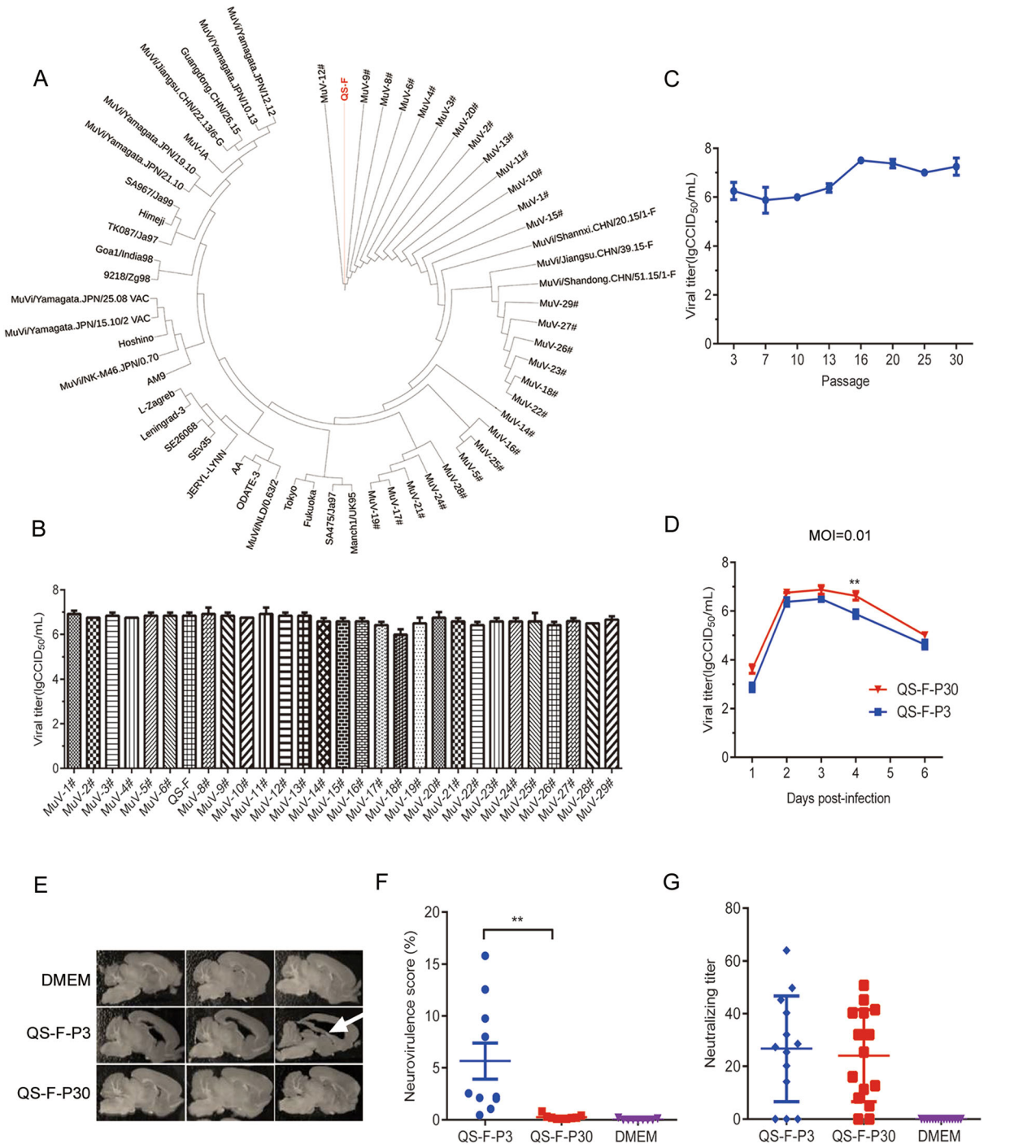

Fig. 1 **A** Phylogenetic tree analysis of amino acid sequence of small hydrophobic protein (SH). QS-F strain was marked with red. Trees were generated by Clustal Omega. **B** Viral titers of 29 MuV strains. Vero cells were infected with 29 strains at an MOI of 0.01, respectively. **C** The multiple growth curve of QS-F every 3–5 passages. Vero cells were infected with QS-F at an MOI of 0.01. **D** The multiple growth curve of QS-F-P3 and QS-F-P30. The virus growth curves were drawn based on the viral titers measured at each indicated time point. **E** Representative sagittal cross-sections of brains from rats inoculated with QS-F-P3, QS-F-P30 and DMEM, respectively after 25 days post infection. Arrow indicates the lateral ventricle. **F** Severity of hydrocephalus in rats inoculated with QS-F-P3, QS-F-P30 and DMEM, respectively. Data are presented as the mean ± SEM (n = 10–16). Asterisks indicate significant differences between the experimental groups as analyzed by unpaired *t* test, data is presented at the mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$;$^{***}P < 0.001$; (N = 15 mice per group). **G** Immunogenicity of QS-F-P3 and QS-F-P30 in mice. Six-week-old Blab/c mice (n = 15) were immunized by i.m. route with 4×10^5 $CCID_{50}$ of QS-F-P3 and QS-F-P30. Blood was collected and sera were used for virus-neutralizing antibodies detection at each time point.

Table 1 Comparison of the nucleotide sequences of QS-F-P3, QS-F-P15 and QS-F-P30.

Position	Gene segment	QS-F-P3		QS-F-P15		QS-F-P30	
		Nucleotide	Amino acids	Nucleotide	Amino acids	Nucleotide	Amino acids
2180	V/P	A	K	A	K	G	E
2300	V/P	C/A	H/T	A	T	A	T
2321	V/P	T/A	S/T	A	T	A	T
2329	V/P	C/A	H/Q	A	Q	A	Q
2360–2361	V/P	TA/GC	Y/A	GC	A	GC	A
2388	V/P	T/A	I/N	A	N	A	N
3355	M	A	E	G	G	G	G
5243	F	C	P	T	L	T	L
5058	F	C	H	C	H	C	H
7395	HN	C	T	T	I	T	I
9315	L	C	S	G	C	G	C
9418	L	A	L	C	L	C	L
14,989	L	G	K	A	K	A	K
15,259	L-3′UTR	A	–	G	–	G	–

(QS-F-P3) and thirty times (QS-F-P30) *in vitro*, Vero cells were infected with either QS-F-P3 or QS-F-P30 at an multiplicity of infection (MOI) of 0.01, the supernatant was harvested at 1, 2, 3, 4 and 6 days post infection (dpi). Virus titration was carried out by 50% cell culture infective dose assay in Vero cells. As Fig. 1D shown, at 4 dpi, the titer of QS-F-P30 was significantly higher than QS-F-P3.

To investigate the gene mutants of QS-F with passaging in Vero cells, the complete genomes of QS-F-P3, QS-F-P15, QS-F-P30, and QS-F-P45 were sequenced and the mutations were summarized in Table 1. Compared to QS-F-P15, only one mutation was occurred on QS-F-P30, and QS-F-P45 had no mutation compared to P30 which means QS-F-P30 has a good genetic stability. Compared to QS-F-P3, 46.1% (6/13) amino acid mutants in QS-F-P30 were found in both V protein and P protein, and other mutants were located in M protein, F protein, HN protein and L protein, respectively. It's reported that V protein is an important viral factor which could reduce the expression of type I interferon and interleukin 6 during infection (Xu *et al.* 2012). In addition, there are evidences demonstrated that V protein is related to the neurotoxicity in infected animals (Xu *et al.* 2012).

To analyze whether QS-F-P30 were highly attenuated with continuous passage in Vero cells, a good animal model should be used. It has been proved that monkey kidney cells are suitable for the attenuation of wild type MuV (Sauder *et al.* 2006). Although monkey neurovirulence test (MNVT) is usually used to assess the neurovirulence of MuV, more and more studies demonstrated that the result of MNVT is not consistent with the neurovirulence level of MuV in humans (Rubin and Afzal 2011). Rubin *et al.* reported that the newborn rat model showed promising results (Rubin *et al.* 2000). In 2005, The scientific workshop on neurovirulence testing of live attenuated viral vaccines in Geneva, the newborn rat model was recommended (Iabs 2006). Here we constructed an improved newborn rat neurovirulence evaluation model. One-day-old Wistar rats (Beijing Vital River Laboratory Animal Technology Co., Ltd.) were intracranially injected (i.c.) with DMEM, QS-F-P3 or QS-F-P30, respectively, at a dose of 100 $CCID_{50}$ in a volume of 10 μL (n = 10–16). The inoculation site was located in the left parietal area of the skull (∼ 2 mm left of midline and midway between the bregma and lambda). The rats were euthanized with chloral hydrate at 25 dpi. Brains were removed and fixed in 4% paraformaldehyde for 24 h. Fixed brains were cut in half in the sagittal plane along the anatomical midline. A single 80-μm-thick section was obtained from each hemisphere at a depth of 0.5–1.0 mm from the surface by Vibratome (LEICA VT1200S, Germany). Then, the neurovirulence score (hydrocephalus severity) is expressed as a percentage, the quotient of the cross-sectional area of the entire brain (excluding the cerebellum) and the cross-sectional area of the lateral ventricle. As shown in Fig. 1E, the sections of rat brain infected with QS-F-P30 or DMEM exhibited no nerve injury. Enlarged ventricle occupied 5.6% of the total brain cross-sectional area in QS-F-P3, and the neurovirulence scores of QS-F-P30 were reduced by 92% compared to the QS-F-P3 (Fig. 1F). It illustrates that QS-F-P30 has been highly attenuated after long term of passages in Vero cells.

To investigate whether the passaging *in vitro* affect the immunogenicity of QS-F *in vivo*, 5 to 6-week-old Balb/c

mice were immunized by the intramuscular (i.m.) route with 4×10^5 $CCID_{50}$ of QS-F-P3 and QS-F-P30 respectively, and boosted at 21 dpi. After 14 days post boost, blood was collected and sera were separated for the neutralization assays. It was noted that the neutralizing antibody titers of mice in QS-F-P30 (26.68 ± 20.05) group were equal with QS-F-P3 (24.04 ± 17.44) group (Fig. 1G), indicating that the immunogenicity of QS-F was not affected by continuous passaging.

In summary, we have developed a novel MuV vaccine candidate, which exhibited low neurovirulence in newborn rats, and had good neutralization activity *in vitro*. Further work will focus on attenuated mechanism of QS-F-P30.

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Compliance with Ethical Standards

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

Human and Animal Rights Statement Animal experiments in this study were approved by the Ethics Committees of Huazhong Agriculture University (Approval Number: HZAUMO-2019-020).

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