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Effect of ORF119 gene deletion on the replication and virulence of orf virus

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Summary. – Orf is a severe infectious disease of sheep and goats caused by orf virus (ORFV). To investigate the role of ORF119 gene of ORFV, we constructed ORFV with deleted ORF119 gene and LacZ as reporter gene (ORFV-Δ119-LacZ) via homologous recombination. The results showed that wild-type ORF-SHZ1 and ORFV-Δ119-LacZ deletion viruses replicated in Vero cells to similar titers. Relative transcriptional levels of virulence genes OVIFNR, GIF, VEGF and VIL-10 of ORFV-Δ119-LacZ deletion virus were slightly but not significantly lower after 24 hr compared with the wtORF-SHZ1 virus. *In vivo* experiments showed that 2-month-old lambs inoculated with ORFV-Δ119-LacZ deletion virus exhibited a similar total clinical score compared with those inoculated with wtORF-SHZ1 virus. Based on these results, we conclude that deletion of the ORF119 gene has no significant effect on ORFV replication and virulence.

Keywords: orf virus; ORF119; gene deletion; replication; virulence

Introduction

Orf is contagious disease caused by orf virus (ORFV), a member of pox virus family, and occurs mainly in sheep and goats (Haig and Mercer, 1998; Bora *et al*., 2012). The primary clinical symptoms of orf include erythema, papules, nodules, vesicles, pustules, ulcers and warty thick crust on lips and tongue, nose, skin and mucosa. Lambs at the age of 2 – 6 month are the most susceptible animals to ORFV. Cases of orf in wild ungulates (deer and reindeer) and humans have also been reported (Klein *et al*., 2005; Bayindir *et al*., 2011; Tryland *et al*., 2013; Karakas *et al*., 2013; Nougairede *et al*., 2013; Zhang *et al*., 2014). In recent years, orf disease has higher prevalence in many countries and regions with sheep industry (Bora *et al*., 2012; Chi *et al*., 2013; Kumar *et al*., 2014; Oem *et al*., 2013; Schmidt *et al*., 2013), especially in some economically underdeveloped areas in China (Li *et al*., 2012). Secondary infection with other microbial pathogens in sick lambs may result in higher mortality, which causes serious economic losses in sheep industry.

ORFV is double-stranded DNA virus with a genome size of approximately 132–140 kb and G, C content of 64%. The genome of ORFV is composed of inverted terminal repeats (ORFs 001-008; ORFs 112-134) and coding sequence in the middle region (ORFs009-111) (Delhon *et al*., 2004). The terminal repeats are variable, while the middle coding region is relatively conserved (McInnes *et al*., 2001; Billinis *et al*., 2012). Genome sequence of ORFV indicates that ORFV genome contains 134 open ORFs and ORF119 is located in the right side of the terminal repeat region. So far, however, the role of ORF119 is unclear. In this study, we constructed ORF119 deficient strain using LacZ as reporter gene in order to elucidate the role of ORF119 in ORFV replication and virulence.

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Abbreviations: ORFV = orf virus; OVIFNR = orf virus interferon-resistance gene; GIF = GM-CSF/IL-2 inhibitory factor; VEGF = vascular endothelial growth factor; VIL-10 = viral interleukin 10; LacZ = beta-galactosidase

Materials and Methods

Virus and cells. Wild-type ORFV SHZ1 strain (ORFV-SHZ1) was isolated from lamb during an orf outbreak at the animal test station of Shihezi University in 2012. African green monkey kidney (Vero)

Table 1. PCR primers used in this study

Primers	Sequence (5' to 3')
P1	GGCTGCAGGACGGCTACTCCCGCTAATG
P2	GGGACGTCCGTATCCAACTGCTTGCCTC
P3	ATGGACTCTCGTCGGCTCGC
P4	GATTGACCGTAATGGGATAG
P5	TGGGATCTGCCATTGTCAGAC
P6	CCTTCATTGTCCGGTTGAGG
DNA polymerase-sense	CAGCATCGACGAGATCGTGG
DNA polymerase-anti-sense	TCGAAGTGGC ACTCTATGTC
OVIFNR-sense	CGAAGGAGCTCGGCATAT
OVIFNR-anti-sense	AACTCGTTGA CCGCACTG
GIF-sense	ATGGCGTGCCTCAGAGTGTT
GIF-anti-sense	GCCGAGTTGTTATCCGCAG
VEGF-sense	ATGAAGTTGCTCGTCGGC
VEGF-anti-sense	GTCTCGCTTACAGGAACA
VIL-10-sense	TGGTGTGTGTTGTGATTATT
VIL-10-anti-sense	GTGTGAGTAGCATACTGTTC

cells were kindly provided by Dr. Wu from Cambridge University and were maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), 100 μg/ml streptomycin,100 U/ml penicillin, 50 μg/ml gentamicin and 2 mmol/l L-glutamine. pSC11 vector was provided by Dr. Wu from Cambridge University.

Construction of shuttle vector. Primer sequences for ORF 119, orf virus interferon-resistance gene (OVIFNR), GM-CSF/IL-2 inhibitory factor (GIF), vascular endothelial growth factor (VEGF) and viral interleukin (VIL-10) genes were designed by Primer Premier 5.0 software based on the whole genome sequence of ORFV (Acc. No. NC_005336.1) (Table 1). Construction strategy of the recombinant shuttle vector pΔ119-LacZ is shown in Fig. 1a. Briefly, genomic DNA from ORFV-SHZ1 was isolated using commercial MiniBEST viral DNA extraction kit (TaKaRa) according the manufacturer's instruction. The homology arm (containing ORFV119 gene) was amplified using primers P1-P2 and inserted to pMD18-T-simple (TaKaRa), generating the recombinant vector pT-ORF119. Vaccinia virus late promoter P11 and reporter gene lacZ from pSC11 vector (Chakrabarti *et al*., 1985) were digested by restriction enzymes *Sma*I and *Pst*I (TaKaRa) and inserted into pT-ORF119 digested by *Not*I and *Sac*I (TaKaRa), generating the recombinant vector pΔ119-LacZ. The shuttle vector pΔ119-LacZ was verified by analysis with restriction enzyme *Pst*I (TaKaRa).

Generation of recombinant ORFV-Δ119-lacZ virus. Vero cells were cultured in 6-well plates containing DMEM with 10% FBS (Life Technologies, USA). Vero cells were infected by 100 $TCID_{50}$ of ORFV-SHZ1 virus. Subsequently, the infected cells were transfected with pΔ119-LacZ shuttle vector using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were cultured at 37°C with 5% CO_2. The supernatant from cells with cytopathic effect (CPE) was freezed and thawed for 3 times and used to infect the new monolayer of Vero cells. The infected cells were over-laid with an agarose-medium mix and after five days of culture, a further agarose layer containing X-gal (Roche) was added to identify viral plaques expressing the lacZ gene. Recombinant clones were subjected to five rounds of plaque purification until all plaques appeared Lac positive. Viral genomic DNA was isolated from the purified plaques using MiniBEST viral DNA extraction kit (TaKaRa) and used as template to identify ORF119 deletion strain using primers P3-P4 and P5-P6 (producing 1,120 and 750 bp fragment, respectively). Insertion of P11 promoter from pSC11 vector and LacZ in the ORF119 deletion site was confirmed by sequencing three times using the dideoxynucleotide chain termination method with an ABI-PRISM 3730xl Autosequencer (Aplied Biosystems, USA). The DNA sequences that were completely identical at least two times were used for the sequence analysis.

One-step growth curve. Vero cells were inoculated with 10 $TCID_{50}$ of ORFV-Δ119-LacZ and ORFV-SHZ1. Virus was harvested after 0, 3, 6, 12, 24 and 48 hr of culturing. The median tissue culture infected dose ($TCID_{50}$) per ml was calculated using a spreadsheet. The replication curves of ORFV-Δ119-LacZ and ORFV-SHZ1 were plotted as titer ($\log_{10}$ $TCID_{50}$/ml) versus time course (hr) post infection. Each experiment was performed with three replicates.

Detection of relative transcriptional levels of virulence gene. Total RNAs were extracted from Vero cells infected with ORFV-Δ119-LacZ or ORFV-SHZ1 for 24 hr using Trizol (Invitrogen). RNAs were reverse transcribed into cDNA with AMV reverse transcriptase kit (TaKaRa), and used as template in real-time RT-PCR using SYBR Premix Ex TaqTM kit (TaKaRa, Japan) on LightCycler 480 (Roche, Switzerland) according to the manufacturers' instructions. DNA polymerase gene of ORFV was used as internal control. Each sample was tested with three replicates, and then the relative transcript levels of OVIFNR, GIF, VEGF and VIL-10 genes were calculated using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

In vivo infection. Two-month-old lambs (n = 12) were divided into three groups with 4 lambs in each group. The first group was inoculated with 0.5 ml (1×10^5 $TCID_{50}$/ml) of ORFV-Δ119-LacZ infected Vero cell culture medium on the lip. The second group was inoculated similarly with ORFV-SHZ1 and the third group was inoculated with sterile saline buffer as control. The groups of lambs were separated and observed for four weeks. After the infection, the development of lesions in ORFV-Δ119-LacZ and ORFV-SHZ1 inoculated lambs was compared daily using a clinical scoring procedure (Nettleton *et al*., 1996).

Statistical analysis. The experimental data were presented as the mean ± standard deviation from three independent experiments and analyzed by SPSS software. The *t*-test was applied to analysis of the difference between the experimental groups and control groups. A p value of less than 0.05 was considered as a statistically significant difference.

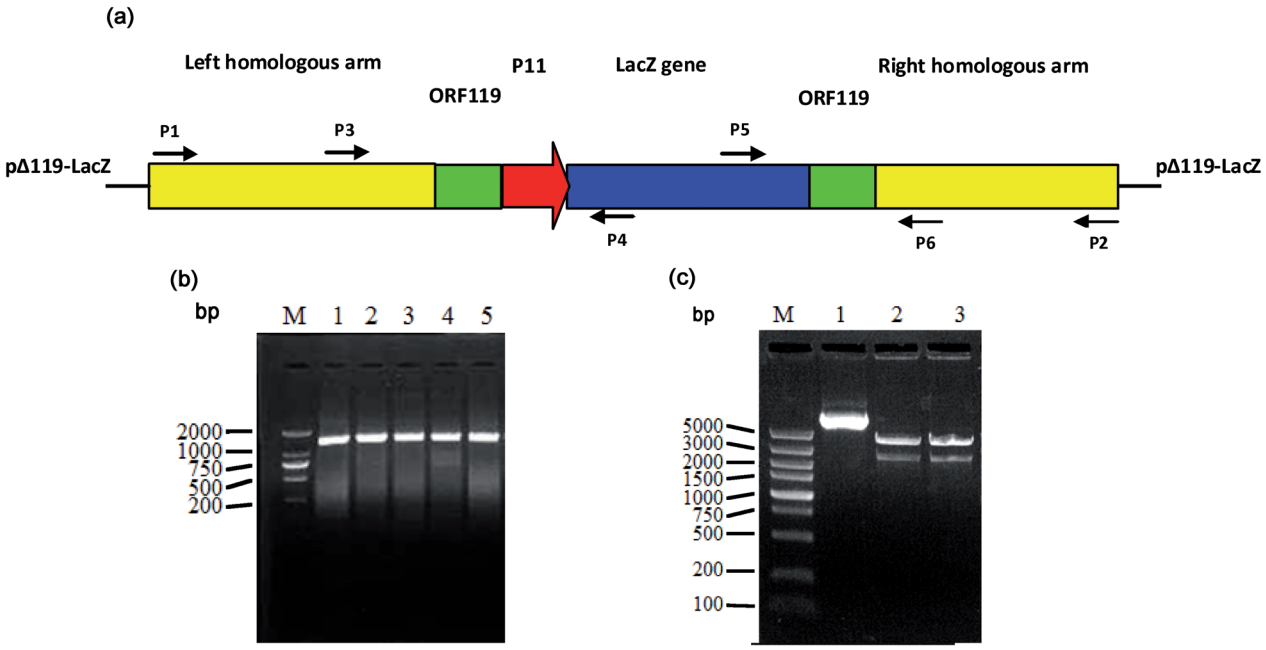

Fig. 1

Construction and identification of the shuttle vector pΔ119-LacZ

(a) Scheme of the shuttle vector pΔ119-LacZ. P11 promoter (red arrow), ORF119 gene (green box), left and right arms for homologous recombination (yellow boxes), LacZ gene (blue box), PCR primers (black arrows). (b) PCR amplification of homology arms containing ORFV119 gene. DNA size marker (DL2000) (lane M), ORFV119 gene and homology arm fragment (lanes 1–5). (Cc Identification of recombinant shuttle vector pΔ119-LacZ digested with *Pst*I. DNA size marker (DL5000) (lane M), pΔ119-LacZ (lane 1), pΔ119-LacZ digested with *Pst*I (lanes 2–3).

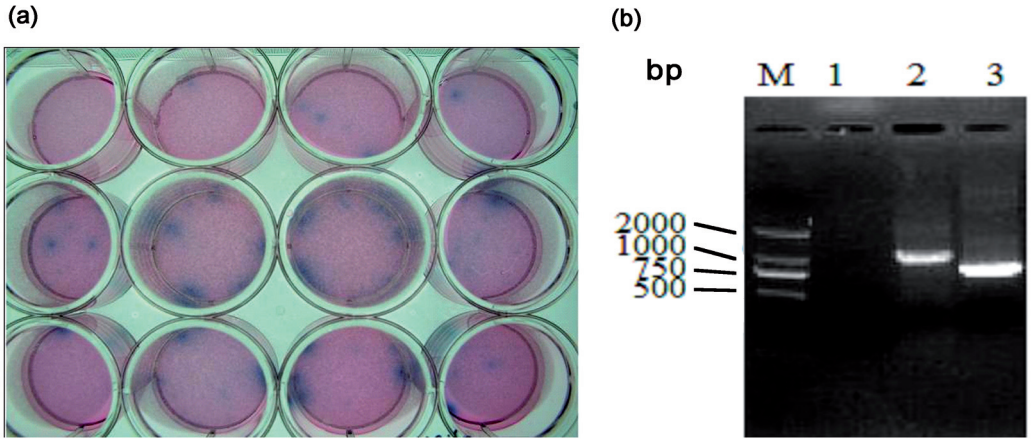

Fig. 2

Generation and identification of recombinant ORFV-Δ119-LacZ

(a) Blue plaques of ORFV-Δ119-LacZ on Vero cells. (b) Identification of ORFV-Δ119-LacZ by PCR. M: DNA size marker (DL2000) (lane M), amplification of ORFV SHZ1 (lane 1), amplification of ORFV-Δ119-LacZ with primers P3-P4 (lane 2), and P5-P6 (lane 3).

Results

The homology arm (containing the upstream, downstream and ORFV119 gene) was amplified with an expected size of 1,475 bp (Fig. 1b). The expected size of the shuttle vector pΔ119-LacZ containing the upstream and downstream sequences of ORF119, P11 promoter and lacZ reporter gene was 7,7 kb, which was confirmed by restriction digestion using *Pst* I (producing 2,700 bp and 5,000 bp bands) (Fig. 1c). ORF119 deficiency strain ORFV-Δ119-LacZ can cause CPE

ATGGACTCTCGTCGGCTCGCCCTTGCCGTTGCCTTTGGAGGCGTCCTCGCCAGCATGACGCAGCGCCGCCGCCTGG

CTTCTCTCATCGCCAGCATCGGCCAACGGTTGATGGGCGGCGACGGCATGCGTCGCGTCGCCGTTCGGTTGATCGA

CCAGCTCATGGCCGGACCCCCGGACATCGACGACGAAGCCTTCCAGCGCGAGATCCGCGTGGGCGTGGGCGAGCTC

CACCCAATCGTTTAGAAAAGGAAGCCTCCTTAAAGCATTTCATACACACAGCAGTTAGTTTTACCACCATTTCAGA

TAATGGAATAAGATTCAAAATATTATTAAACGGTTTACGTTGAAATGTCCCATCGAGTGCGGCTACTATAACTATT

TTTCCTTCGTTTGCCATACGCTCACAGAATTCCCGGGGATCCGTCACTGTTCTTTATGATTCTACTTCCTTACCGT

GCAATAAATTAGAATATATTTTCTACTTTTACGAGAAATTAATTATTGTATTTATTATTTATGGGTGAAAAACTTA

CTATAAAAAGCGGGTGGGTTTGGAATTAGTGATCAGTTTATGTATATCGCAACTACCGGGCATATGGCTATCGACA

TCGAGAACATTACCCACATGATAAGAGATTGTATCAGTTTCGTAGTCTTGAGTATTGGTATTACTATATAGTATAT

AGATGTCTAGAAGCGATGCTACGCTAGTCACAATCACCACTTTCATATTTAGAATATATGTATGTAAAAATATAGT

AGAATTTCATTTTGTTTTTTTCTATGCTATAAATGAATTCCTCGAGGGATCCCGTCGTTTTACAACGTCGTGACTG

GGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAG

GCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGCGCTTTGCCTGGTTTCCGGCACCAG

AAGCGGTGCCGGAAAGCTGGCTGGAGTGCGATCTTCCTGAGGCCGATACTGTCGTCGTCCCCTCAAACTGGCAGAT

GCACGGTTACGATGCGCCCATCTACACCAACGTGACCTATCCCATTACGGTCAATC

Fig. 3

The nucleotide sequence of left homologous arm fragment of the recombinant ORFV-Δ119-LacZ

The primer sequence is double-underlined (red); left homologous arm is underlined (blue), and the remaining part is the inserted gene sequences (black).

TGGGATCTGCCATTGTCAGACATGTATACCCCGTACGTCTTCCCGAGCGAAAACGGTCTGCGCTGCGGGACGCGCG

AATTGAATTATGGCCCACACCAGTGGCGCGGCGACTTCCAGTTCAACATCAGCCGCTACAGTCAACAGCAACTGAT

GGAAACCAGCCATCGCCATCTGCTGCACGCGGAAGAAGGCACATGGCTGAATATCGACGGTTTCCATATGGGGATT

GGTGGCGACGACTCCTGGAGCCCGTCAGTATCGGCGGAATTCCAGCTGAGCGCCGGTCGCTACCATTACCAGTTGG

TCTGGTGTCAAAAATAATAATAACCGGGCAGGGGGGATCCCCGACCTGCAGCCAAGCTCCGAATTCAACAATGTCT

GGAAAGAACTGTCCTTCATCGATACCTATCACGGAGAAATCTGTAATTGATTCCAAGACATCACATAGTTTAGTTG

CTTCCAATGCTTCAAAATTATTCTTATCATGCGTCCATAGTCCCGTTCCGTACCCCAGCCCGCGCCCCTGGCGATC

ACGCCACAGAACGCGTTCATGTTCGTGCCGCAAAGCAGCCACGTGCACGTGGACGAGAGCGTGGACCCGTTCTTCG

GCATGAGCCCCTCCATCTTCGGGCGCAACATCCCCCTTCAGCCGCCCGAGGAGCTGCTGAGCGACTACGACCCGCT

CATGAGCCAGGCCGCCCTACCTCACCCACACCCACTCACACCAACACCTCAACCGGACAATGAAGG

Fig. 4

The nucleotide sequence of right homologous arm fragment of the recombinant ORFV-Δ119-LacZ

The primer sequence is double-underlined (red); the right homologous arm is underlined (blue), and the remaining part is the LacZ gene sequence (black).

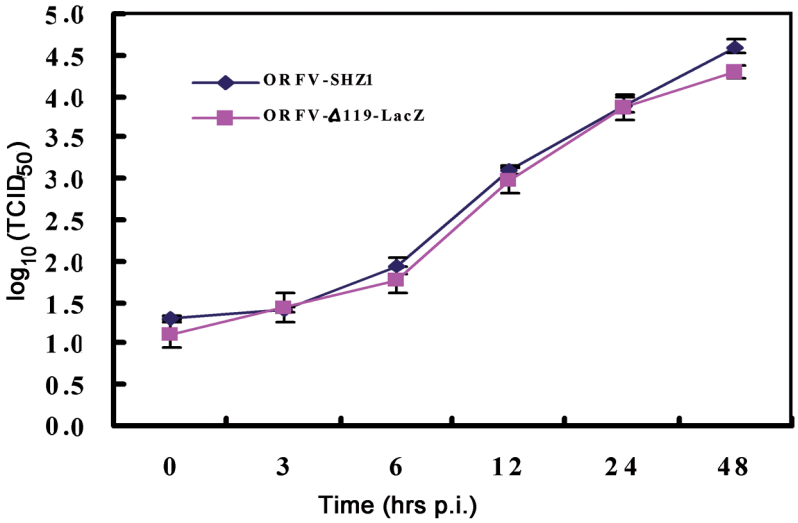

Fig. 5

Replication curves of ORFV-SHZ1 and ORFV-Δ119-LacZ in Vero cells
Data represent averages from three independent experiments. ORFV-SHZ1 (diamonds) ORFV-Δ119-LacZ (squares), SD (error bars).

in Vero cells and produce blue plaques (Fig. 2a), indicating that the recombinant virus expresses LacZ gene. PCR using primers P3-P4 and P5-P6 yielded bands of expected size of 1,120 bp and 750 bp from ORFV-Δ119-LacZ, but failed to amplify any band from ORFV-SHZ1 (Fig. 2b). Sequence analysis showed that PCR product amplified with P3-P4 primers contained partial sequences of the upstream ORF119 gene, P11 promoter and LacZ sequences (Fig. 3). The PCR product amplified by primer P5-P6 contained ORF119 downstream and inserted sequences (Fig. 4), indicating that ORF119 deletion strain (ORFV-Δ119-LacZ) was successfully constructed.

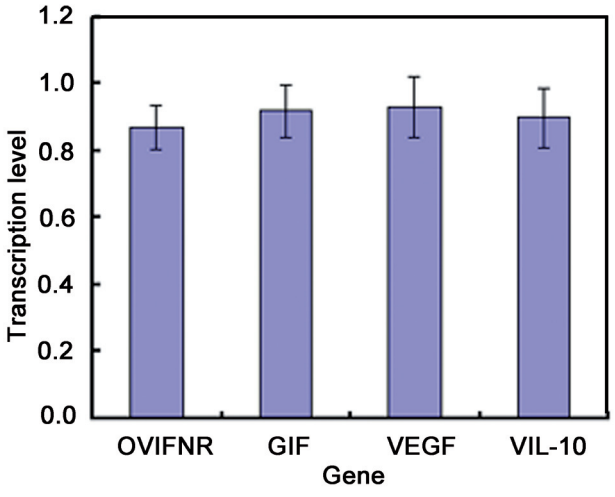

Fig. 6

Relative expression levels of OVIFNR, GIF, VEGF and VIL-10 genes of ORFV-Δ119-LacZ in Vero cells 48 hr post infection

To investigate the replicating ability of ORF119 gene-deleted mutant strain, a one-step growth curve was conducted. After 0, 3, 6, 12, 24 and 48 hr of Vero cell infection, $TCID_{50}$ of ORFV-Δ119-LacZ was slightly lower than that of ORFV-SHZ1. However, statistics showed that there was no significant difference between ORFV-SHZ1 and ORFV-Δ119-LacZ ($P>0.05$). The replication curve of ORFV-Δ119-LacZ was similar to that of ORFV-SHZ1 (Fig. 5), suggesting that deletion of ORF119 has no significant effect on the virus replication in Vero cells.

The relative transcriptional levels of virulence genes including OVIFNR, GIF, VEGF and VIL-10 in the ORF119 deletion virus were slightly, but not significantly ($P>0.05$), lower compared to those in the wild-type virus (Fig. 6), suggesting that ORF119 does not affect the expression of virulence genes of ORFV.

Papules and pustules occurred in the mouth and lips of lambs infected with both ORFV-SHZ1 and ORFV-Δ119-LacZ strain for one week. After two weeks of infection, scabs gradually occurred in both ORFV-SHZ1 and ORFV-Δ119-LacZ-infected lambs (Fig. 7). In the lambs inoculated with saline buffer, no pathological changes were observed. Lambs inoculated with ORFV-Δ119-LacZ had no significant difference ($P>0.05$) in total clinical scores in period of observation after infection than that of lambs inoculated with ORFV-SHZ1 (Fig. 8). This suggests that ORF119 has no significant effect on the virulence *in vivo*.

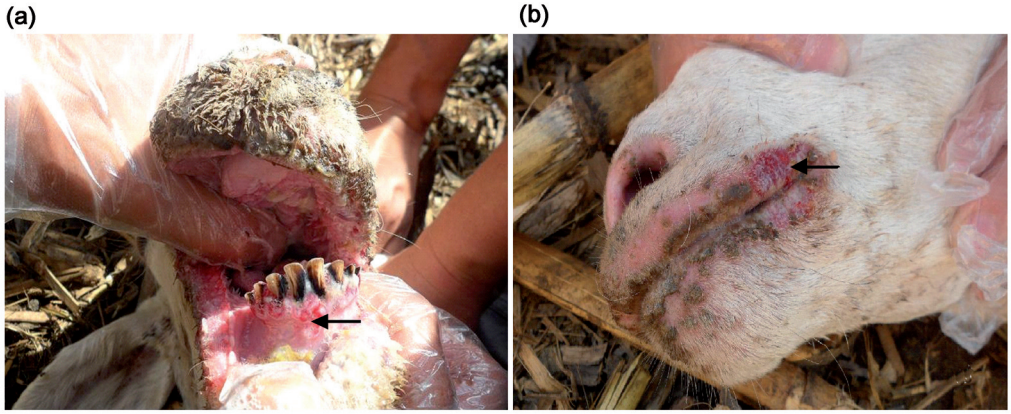

Fig. 7

Clinical symptoms of sheep inoculated with ORFV-Δ119-LacZ
Ulceration of gingival mucosa (a) and the lip (b, arrow).

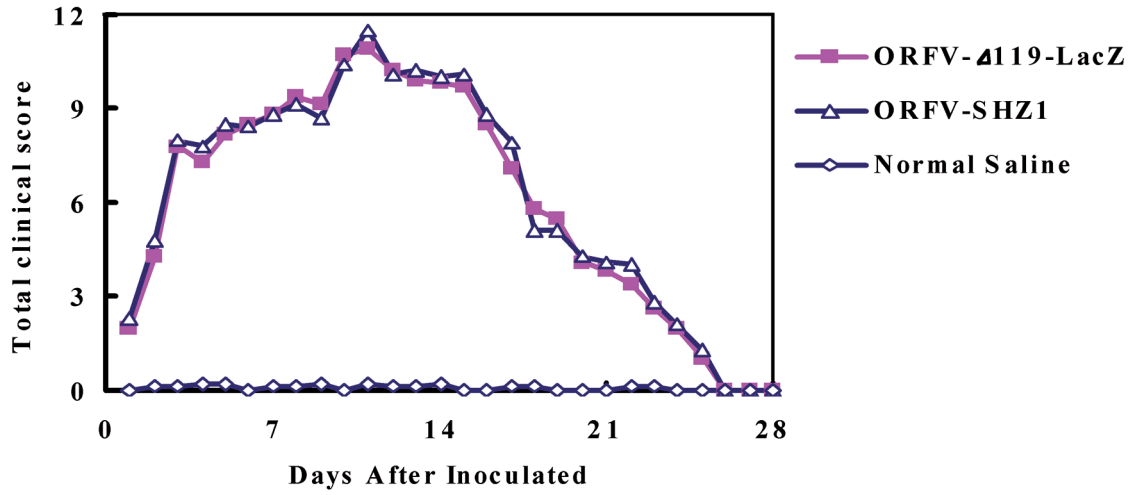

Fig. 8

Total clinical scores in period of observation after inoculation with ORFV-SHZ1 and ORFV-Δ119-LacZ
Data show 2-month-old lambs inoculated with ORFV-Δ119-LacZ deletion virus which exhibited a similar total clinical score compared with those inoculated with wtORF-SHZ1 virus. ORFV-SHZ1 (triangles), ORFV-Δ119-LacZ (squares), saline buffer (diamonds).

Discussion

Orf is an acute, highly contagious zoonotic disease (Bayindir *et al.*, 2011; Nougairede *et al.*, 2013). Prevention and control of the disease cannot only improve the economic efficiency in sheep industry, but also has important public health implications. Although whole genome sequences for four strains of ORFV (OV-SA00, OV-IA82, NZ2 and D1701) have been deposited in the GenBank, functions of many genes are still poorly understood (Delhon *et al.*, 2004; Friebe *et al.*, 2011). Comparison of the whole genome sequence of sheep ORFV isolate (OVIA82) and goat isolate (OV-SA00) with bovine papular stomatitis virus isolate indicated that ORFV contained 132–134 ORFs (Delhon *et al.*, 2004). Furthermore, virulence and immune regulation-related genes were concentrated at the terminal inverted repeats (ITRs) region of the ORFV genome (Haig and Fleming, 1999; McInnes *et al.*, 2001; Friebe *et al.*, 2011). At present, only some ORFV virulence factors have been confirmed, which include viral interferon inhibitory protein (OVIFN, ORF020) (Haig *et al.*, 1998), chemokine binding protein (CBP, ORF112) (Seet *et al.*, 2003), GM-CSF/IL-2 inhibitory

factor (GIF, ORF117) (Deane *et al.*, 2000), viral interleukin 10 (VIL-10, ORF127) (Fleming *et al.*, 1997; Chan *et al.*, 2006) and vascular endothelial growth factor (VEGF, ORF132) (Lyttle *et al.*, 1994; Savory *et al.*, 2000). Westphal *et al.* (2009) utilized vaccinia and ORFV virus recombinant technology to study the biological function of ORF125 and demonstrated that ORF125 is capable of inhibiting the activity of Bcl-2 and apoptosis in the host cells to facilitate viral replication. Diel *et al.*, found that ORF121-encoded protein can inhibit NF-κB signaling pathway, thereby inhibiting the secretion of a number of important cytokines and immune response of host cells (Diel *et al.*, 2011). However, the function of many genes encoded by the terminal ITRs (ORFs 001-008 and 112–134) has not been elucidated.

At present, ORFV is proving useful both as an immunomodulator and as a vector for the expression of foreign antigens in non-permissive species (Amann *et al.*, 2013; Fischer *et al.*, 2003; Rohde *et al.*, 2011, 2013; Tan *et al.*, 2012). To understand the function of ORFV replication and virulence-related genes is the premise to develop novel vaccine vectors. However, so far, the role of ORFV119 has not been reported. In this study, we generated ORF119 deletion strain by homologous recombination and demonstrated that ORF119 does not play an important role in the viral replication in Vero cells. Furthermore, the expression of virulence related genes in the ORF119 deficiency strain was not significantly different from those of the wild type strain. *In vivo* studies confirmed that both ORF119 deletion and wild type ORFV strains can cause disease in lambs, suggesting that ORF119 is not required for viral replication in host cells and virulence both *in vitro* and *in vivo*.

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