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Identification and phylogenetic analysis of a sheep pox virus isolated from the Ningxia Hui Autonomous Region of China

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ABSTRACT. An outbreak of sheep pox was investigated in the Ningxia Hui Autonomous Region in China. Through immunofluorescence testing, isolated viruses, polymerase chain reaction identification, and electron microscopic examination, the isolated strain was identified as a sheep pox virus. The virus was identified through sequence and phylogenetic analysis of the P32 gene, open reading frame (ORF) 095, and ORF 103 genes. This study is the first to use the ORF 095 and ORF 103 genes as candidate genes for the analysis of sheep pox. The results showed that the ORF 095 and ORF 103 genes could be used for the genotyping of the sheep pox virus.

Key words: Capripoxvirus; Small-tailed Han sheep; ORF 095 gene; ORF 103 gene; P32 gene; Phylogenetic analysis

INTRODUCTION

Capripoxviruses (CaPVs) cause goat pox, sheep pox, and lumpy skin disease, which have negative economic effects on herds of goat, sheep, and cattle, respectively. Goat pox and sheep pox are acute, febrile, contagious diseases (Mangana-Vougiouka et al., 1999; Babiuk et al., 2008). Sheep pox virus (SPPV) and goat pox virus (GTPV) affect both sheep and goats and occur in Africa, Middle East, and Asia (Le Goff et al., 2009). These viruses cause systemic disease in all ages of sheep and goats but affect the young most severely. In contrast, the lumpy skin disease virus affects cattle and it is not currently found in China. Sheep and goat pox are old diseases in China, with reports dating to as early as the 1st century AD (Yan et al., 2010).

SPPV and GTPV cause systemic disease in sheep and goats that is characterized by fever, lesions in the respiratory and gastrointestinal tracts, lymph node enlargement, hair loss and less gross parts of the skin, and mucous membrane pimples and herpes. The incubation period is 2-12 days with an average of 6-8 days. Before showing multifocal necrotic lesions in the skin, sheep infected with the virus usually have hyperthermia symptoms, with temperatures ranging between 40 and 42°C, loss of appetite, depressed activity, conjunctival flush, viscous or purulent runny nose, elevated heart rate, and rapid breathing. These sheep display a 1- to 4-day course of multifocal necrotic lesions (Kitching and Taylor, 1985; Black, 1995; Kitching, 2000, 2003). In goats, the pox tends to occur on the breast skin surface, followed by papules gradually increasing into gray blisters containing clear fluid (Smith and Sherman, 2009). At this stage, temperature in diseased sheep tends to be normal. Because leukocytes rupture or their contents dry up, multi-focal necrotic lesions may recur and form brown scabs. The diagnosis of sheep and goat pox has traditionally used characteristic clinical signs with polymerase chain reaction (PCR) for virus isolation. PCR has provided a sensitive and powerful technique for identifying this infection (Mangana-Vougiouka et al., 2000; Hosamani et al., 2004; Zheng et al., 2007). In this study, we used published molecular tools to identify the outbreak of sheep pox virus, and 2 genes were used for the first time to characterize different CaPVs.

MATERIAL AND METHODS

Disease outbreaks

A natural outbreak of SPPV among sheep occurred during November 2011 on a farm with 33 small-tailed Han sheep and 56 local sheep for meat production. The herd included 20 breeding sheep, 43 fatting sheep, and 26 lambs aged 4-6 months. The farm was located in Ningxia Hui Autonomous Region of China. The outbreak occurred because the breeding sheep were not sold through quarantine. Of the 26 breeding sheep in the flock, 10 developed variola lesions over the whole body. The morbidity and mortality of the disease was 26 (29.2%) and 13 (14.6%) in 89 samples, respectively.

Indirect immunofluorescence assay (IFA) and viruses isolated

The multifocal necrotic lesions collected from the lungs were ground in 0.01 M phosphate-buffered saline, pH = 7.4, and repeatedly frozen and thawed 3 times. Then, the homogenized samples were centrifuged at 3000 rpm for 20 min at 4-8°C. The clarified supernatants

were used to inoculate a confluent monolayer of lamb testis (LT) cells grown on 6-well cell culture plates and in a 25-cm^2 cell culture bottle (Costor, Corning Incorporated, USA) in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1.6 mg/L ampicillin, and 100 mg/mL streptomycin. Incubated cells were kept at 37°C with a CO_2 incubator supplying 95% air and 5% CO_2. Normal control cells were maintained in a similar manner. The cells were observed daily using an inverted microscope for any cytopathic effects (CPEs). Cells with CPEs were subjected to an IFA test with rabbit anti-GTPV polyclonal antibodies (laboratory preparation diluted 1:100). The secondary antibody used was fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (1:500 dilution in Evans blue). After immunostaining, the control and inoculated cells were observed under a fluorescence microscope. GTPV in the cell culture bottle was then harvested and stored at -20°C (Zhao et al., 2010).

Electron microscopy

The multiplied virus samples were prepared as described above and resuspended in 2% phosphotungstic acid solution. A drop of this suspension was applied to a carbon-coated formvar film on a 400-mesh copper grid. The grid was examined with a JEOL 1400 transmission electron microscope (Tokyo, Japan) to investigate the virus surface structure. Cells free of CaPV infection were used as a negative control.

Extraction of genomic DNA

The supernatant of LT cells on which CPEs formed stably was collected for viral DNA extraction using a Minibest Viral DNA Extraction Kit (Takara, Dalian, China) according to manufacturer instructions and then used as a template in PCR.

PCR amplification

The open reading frames (ORFs) of the P32, ORF 095, and ORF 103 genes were amplified via PCR from DNA extracted from CPE-positive cell cultures (Tulman et al., 2002). Primers for the specific amplification of the complete P32, ORF 095, and ORF 103 gene sequences were designed using the Oligo 6.0 software based on a published SPPV (10700-99 strain) genomic sequence available in the GenBank (accession No. AY077832). The primers were custom synthesized by the Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. (China). The 2 primer sets were specific for the P32 gene (forward primer: 5'-GCGGATCCTTTCTACAGGCT-3'; reverse primer: 5'-GCGGATCCACTATATATACGT-3'); ORF 095 gene (forward primer: 5'-ATGGACTTCATGAAAAAATATACT-3'; reverse primer: 5'-TTTGCTGTTATTATCATCCAG-3'), or ORF 103 gene (forward primer: 5'-ATGTCTGATAAAAAATTATCTCG-3'; reverse primer: 5'-ATCCATACCATCGTCGATAG-3'). PCR was performed in a 50-μL reaction mixture that contained 25 μL 10X buffer I, 500 mM of each deoxyribonucleotide triphosphate, 0.4 mM of each oligonucleotide primer, 200 ng of each DNA sample, and 1.0 U LA Taq DNA polymerase with 10X buffer (Takara). PCR amplification proceeded with an initial denaturation step of 94°C for 5 min followed by 35 cycles at 94°C for 1 min, 52°C for 45 s, 72°C for 1 min, and a final extension of 72°C for 10 min. Amplicons were visualized via electrophoresis on a 1.0% agarose gel and documented with a gel documentation system.

Cloning and DNA sequencing

The PCR products of the P32, ORF 095, and ORF 103 genes were then cloned into a pMD-20T vector (Takara) and transformed into *Escherichia coli* JM 109. At least 4 positive clones of each selected amplification were sequenced at the Shanghai Sangon Biological Engineering Technology and Services Co., Ltd.

Phylogenetic analysis

Comparison of the isolated sequences with those of Poxviridae available in the GenBank database was performed using the online Basic Local Alignment Search Tool program. Sequence identities of nucleotides between the isolated and NCBI sequences were analyzed using Clustal W. The nucleotide sequences deduced were assembled into a multiple sequence alignment. A phylogenetic tree derived from the nucleotide sequences deduced was constructed for the parapoxviruses by using the neighbor-joining method with the Molecular Evolutionary Genetics Analysis software (version 5.0) (Zhou et al., 2012).

RESULTS

To detect whether the virus antigen existed, we performed an IFA test on CPE-positive LT cells. Virus-specific green fluorescence was found in the cytoplasm of infected cells (Figure 1A). None of the control cells was stained (Figure 1B). After 4-5 days post-infection, the CPEs of the first generation of viruses on the LT cells were observed. They were characterized by ballooning, increased refractivity, and detachment of the cells from the surface of the flask. With the increase in numbers of virus passages, CPE formation became stable (Figure 1C). Compared with infected LT cells, non-infected LT cells displayed normal growth on microscopy analysis (Figure 1D). After the viruses underwent 6 passages in LT cells, CPEs were prominent (48 h post-infection). The viruses were harvested 5-6 days post-infection (Yan et al., 2012).

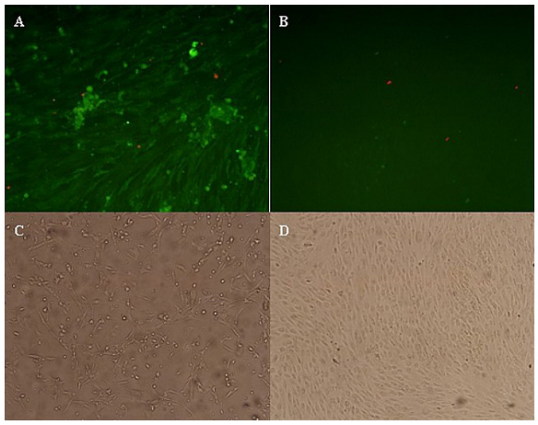

Figure 1. Fluorescence and white light microscopy analysis of virus-infected and uninfected lamb testis (LT) cells. **A.** Fluorescence microscopy analysis of infected LT cells; **B.** fluorescence microscopy analysis of uninfected LT cells; **C.** white light microscopy analysis of uninfected LT cells; **D.** white light microscopy analysis of LT cells infected with the virus.

The supernatant of CPE-positive cultures displayed the characteristic parapoxvirus virions on electron microscopic examination. The virions were approximately 200 nm long and 150 nm wide (Figure 2A). To gain further information from the viral sequences, we implemented a PCR to diagnose CaPV infection from field specimens of infected animals. To confirm the causative agent, we amplified the full length of the P32, ORF 095, and ORF 103 genes via PCR. Three specific products with the expected size (Figure 2B), including the entire region of the P32 (987 bp), ORF 095 (483 bp), and ORF 103 (570 bp) genes, were amplified from the sample extracted from the CPE-positive LT cells. The purified PCR products were cloned into the pMD20-T (TaKaRa) vector, and the positive clones were sequenced.

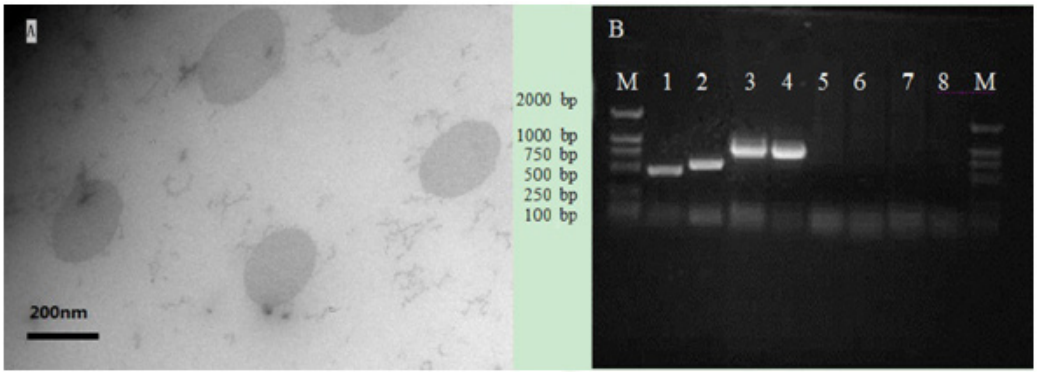

Figure 2. A. Electron microphotograph showing the characteristic morphology of Capripoxvirus virion (bar = 200 nm). **B.** Amplification of major gene by PCR. *Lane M* = DL2000 DNA marker, *lane 1* = Capripoxvirus ORF 095 gene (483 bp); *lane 2* = ORF 103 gene (570 bp); *lane 3* = P32 gene (987 bp); *lane 4* = positive control; *lanes 5* to *8* = untreated control group.

DISCUSSION

CaPVs have been listed as Class A animal diseases by Office International Des Epizooties (OIE), which are endemic in Mongolia, Greece, Kazakhstan, Azerbaijan, and several parts of China and of great economic significance to farmers. In addition, they are a major constraint to the international trade in livestock and their products. The increasing legal and illegal trades in live animals provide avenues for further spread of this disease. CaPVs could also be used as an economic bioterrorism agent. In the last decade, most of the case reports of CaPVs have originated in northwest China, followed by central and southern China (Yan et al., 2010).

The Ningxia Hui autonomous region is located in northwest China and is the main sheep-breeding region of China. According to official veterinary bulletins and published literature, CaPVs are frequent occurrences endemic to this region. In the disease outbreak of the infected sheep in this study, the skin over whole body of the animals had extensive cutaneous lesions, reddish macules and papules 0.5-2 cm in diameter, and pyrexia of 40-42°C. The hair was erect over the skin lesions, the skin was thickened, and crusts of exuded serum were present on the surface. Healing left an ulcer and then a scar after the full skin thickness sloughed (Figure 3).

Figure 3. Photograph of sheeps with severe, proliferative, Capripoxvirus lesions in the skin.

CaPVs have traditionally been considered host-specific, causing outbreaks in a preferred host. However, recent records have indicated that some CaPV strains infect both sheep and goats (Bhanuprakash et al., 2006, 2010). Information based only on the host animal species from which the strain was first isolated is inadequate to identify CaPVs, and owing to the very close antigenic relationship among CaPVs, conventional serological methods cannot distinguish SPPV and GTPV. Therefore, effective molecular techniques are necessary for unequivocal strain differentiation. The most frequently used technique is still PCR, and the P32 gene has been developed for the differentiation of CaPVs (Hosamani et al., 2004). The G protein-coupled receptor gene (Le Goff et al., 2009) and the 30-kDa RNA polymerase subunit gene (Lamien et al., 2011) are also being considered for use in PCR testing of CaPVs (Yan et al., 2012).

In this study, a field CaPV was successfully isolated from diseased sheep in 2011 in the Ningxia Hui Autonomous Region of China. According to sequence analysis of the P32, ORF 095, and ORF 103 genes, the virus strain was an SPPV with a certain degree of variation compared with other isolates. The identities of the nucleotide sequences between the ORF 095 and ORF 103 genes of the isolated strain and GTPV downloaded from NCBI was 96.4 and 95.4%, respectively. The identities of the nucleotide sequences between the ORF 095 and ORF 103 genes of the isolated strain and SPPV downloaded from NCBI was 99.6-99.8 and 98.2%, respectively. Using the P32 gene, we established a PCR-restriction fragment length polymorphism method for a clear distinction between GTPV and SPPV (Tulman et al., 2002).

Using the phylogenetic analysis of the P32 gene, we concluded that CaPVs comprise 3 groups: GTPV, SPPV, and lump skin disease (Figure 4). Nevertheless, a single gene is insufficient to elucidate the genetic relatedness of CaPVs owing to the enormous size of their genomes (Lamien et al., 2011). Multi-gene analysis will greatly improve the accuracy of the information and contribute to research on the epidemiology of SPPV in China. This study is the first to use ORF 095 and ORF 103 gene sequence analysis

to distinguish CaPVs. The results of this study showed that the these CaPV genes may be effective in distinguishing GTPV and SSPV (Figure 5). Comparing the ORF 095 and ORF 103 genes of SSPV and GTPV, we found that the base pair GC is more abundant in GTPV compared with that in SSPV. CaPV is a highly conserved virus. The 2 genes may be recognized as new markers to distinguish GTPV and SSPV. Furthermore, the results of this study are important for the prevention and control of SSPV and will be helpful in the development of a vaccine.

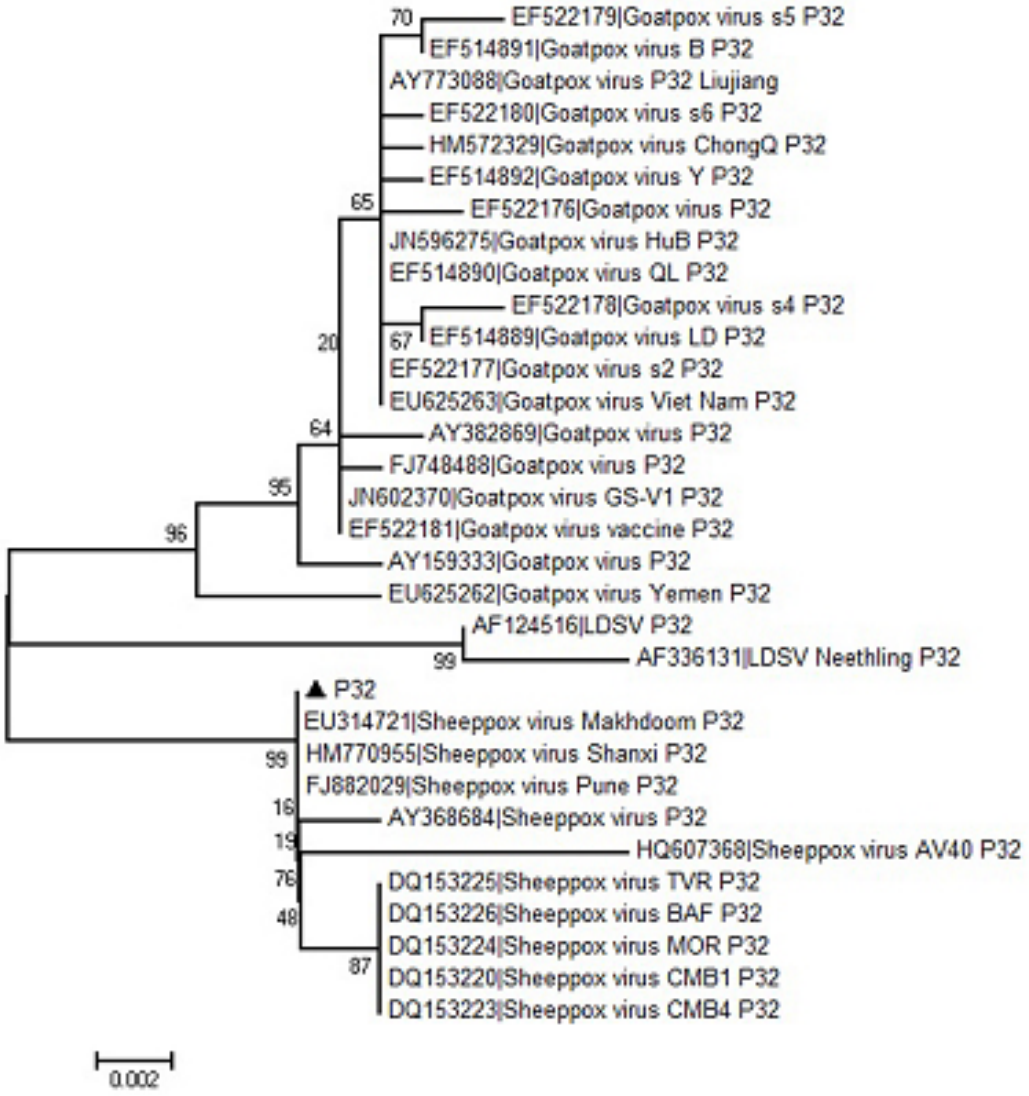

Figure 4. Phylogenetic analysis of different Capripoxvirus based on deduced nucleotide sequence of the P32 gene. The phylogenetic relationship was constructed using the neighbor-joining program of MEGA (version 5.0) software.

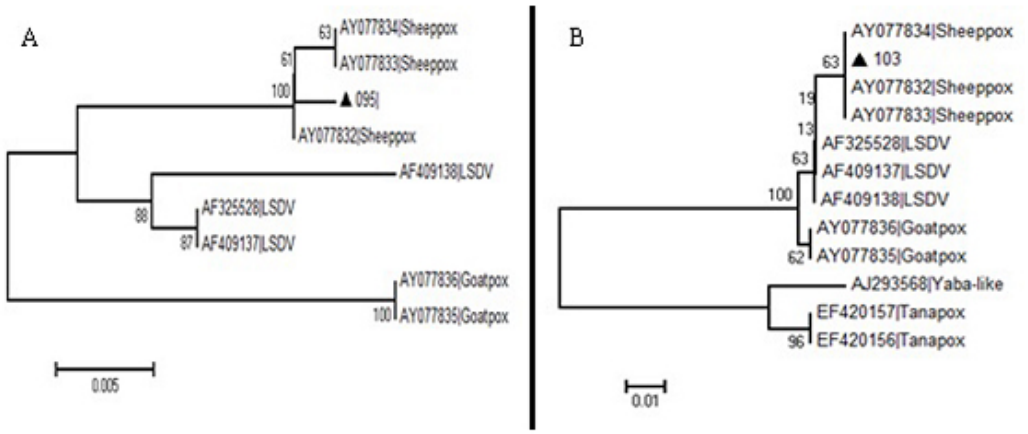

Figure 5. A. Phylogenetic analysis of different Capripoxvirus based on deduced nucleotide sequence of the ORF 095 gene. **B.** Phylogenetic analysis of different Capripoxvirus based on deduced nucleotide sequence of the ORF 103 gene. The phylogenetic relationship was constructed using the neighbor-joining program of MEGA (version 5.0) software.

In conclusion, an outbreak of sheep pox was identified in Ningxia, China, using published molecular tools. The results showed that molecular techniques based on the P32, ORF 095, and ORF 103 genes are efficient for the characterization and differentiation of CaPVs. This finding may provide new information on the epidemiology of SSPV in China.

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REFERENCES

Babiuk S, Bowden TR, Boyle DB, Wallace DB, et al. (2008). Capripoxviruses: an emerging worldwide threat to sheep, goats and cattle. *Transbound. Emerg. Dis.* 55: 263-272.

Bhanuprakash V, Indrani BK, Hosamani M and Singh RK (2006). The current status of sheep pox disease. *Comp. Immunol. Microbiol. Infect. Dis.* 29: 27-60.

Bhanuprakash V, Venkatesan G, Balamurugan V, Hosamani M, et al. (2010). Pox outbreaks in sheep and goats at Makhdoom (Uttar Pradesh), India: evidence of sheeppox virus infection in goats. *Transbound. Emerg. Dis.* 57: 375-382.

Black DN (1995). Capripoxvirus-based multivaccines. *Dev. Biol. Stand.* 84: 179-182.

Hosamani M, Mondal B, Tembhurne PA, Bandyopadhyay SK, et al. (2004). Differentiation of sheep pox and goat poxviruses by sequence analysis and PCR-RFLP of P32 gene. *Virus Genes* 29: 73-80.

Kitching RP (2000). OIE List A disease as a constraint to international trade. *Ann. N. Y Acad. Sci.* 916: 50-54.

Kitching RP (2003). Vaccines for lumpy skin disease, sheep pox and goat pox. *Dev. Biol.* 114: 161-167.

Kitching RP and Taylor WP (1985). Transmission of capripoxvirus. *Res. Vet. Sci.* 39: 196-199.

Lamien CE, Le Goff C, Silber R, Wallace DB, et al. (2011). Use of the Capripoxvirus homologue of Vaccinia virus 30kDa RNA polymerase subunit (RPO30) gene as a novel diagnostic and genotyping target: Development of a classical PCR method to differentiate Goat poxvirus from Sheep poxvirus. *Vet. Microbiol.* 149: 30-39.

Le Goff C, Lamien CE, Fakhfakh E, Chadeyras A, et al. (2009). Capripoxvirus G-protein-coupled chemokine receptor: a

host-range gene suitable for virus animal origin discrimination. *J. Gen. Virol.* 90: 1967-1977.
Mangana-Vougiouka O, Markoulatos P, Koptopoulos G, Nomikou K, et al. (1999). Sheep poxvirus identification by PCR in cell cultures. *J. Virol. Methods* 77: 75-79.
Mangana-Vougiouka O, Markoulatos P, Koptopoulos G, Nomikou K, et al. (2000). Sheep poxvirus identification from clinical specimens by PCR, cell culture, immunofluorescence and agar gel immunoprecipitation assay. *Mol. Cell Probes* 14: 305-310.
Smith MC and Sherman DM (2009). Goat Medicine. Wiley-Blackwell, Ames.
Tulman ER, Afonso CL, Lu Z, Zsak L, et al. (2002). The genomes of sheeppox and goatpox viruses. *J. Virol.* 76: 6054-6061.
Yan X, Wu G, Li J, Zhu H, et al. (2010). The analysis of capripox' epidemic situation in China. *Chin. Agric. Sci. Bull.* 26: 6-9.
Yan XM, Chu YF, Wu GH, Zhao ZX, et al. (2012). An outbreak of sheep pox associated with goat poxvirus in Gansu province of China. *Vet. Microbiol.* 156: 425-428.
Zhao K, Song D, He W, Lu H, et al. (2010). Identification and phylogenetic analysis of an Orf virus isolated from an outbreak in sheep in the Jilin province of China. *Vet. Microbiol.* 142: 408-415.
Zheng M, Liu Q, Jin N, Guo J, et al. (2007). A duplex PCR assay for simultaneous detection and differentiation of Capripoxvirus and Orf virus. *Mol. Cell Probes* 21: 276-281.
Zhou T, Jia H, Chen G, He X, et al. (2012). Phylogenetic analysis of Chinese sheeppox and goatpox virus isolates. *Virol. J.* 9: 25.

