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Original paper

Phylogenetic analysis of Mongolian sheep and goat pox viruses

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Abstract

Sheep and goat pox are caused by sheep pox virus (SPPV) and goat pox virus (GTPV), members of *Capripoxvirus* genus, *Poxviridae* family. SPPV and GTPV damage host animal's wool and skin and reduce production of mutton and milk. Because of morbidity and mortality of the diseases, they bring huge economic burden to the country. Main goal was to compare Mongolian sheep pox, goat pox sequences with other strains that were registered in Genebank.

In this study, two SPPV and two GTPV field strains from Mongolia and Perego M strain (Biocombinat SOI, Mongolia), Russian and Chinese alive vaccine strains were used. The common DNA extraction method was used and samples were amplified on a nested polymerase chain reaction (nested-PCR) which amplify the full p32 gene of Capripoxvirus. The primers were designed based on the conserved sequences just outside of the p32 gene of SPPV or GTPV. By applying this method to the sheep and goat samples, suspected with SPPV and GTPV infection in Mongolia, the nested-PCR products were obtained from all samples on the predicted size, and the presence of SPPV and GTPV were confirmed via full length sequence analysis of P32 gene. Sequence comparison was performed using the online BLAST program. Sequence identities of nucleotides were analyzed using MUSCLE algorithm. A phylogenetic tree derived from nucleotide sequences was constructed for the *Capripoxvirus* using the neighbor joining method of MEGA (version X) software. Based on the phylogenetic tree, the Mongolian sheep pox virus, 2017 clustered together with Zabaikalsk strain and Perego strain (Biocombinat SOI, Mongolia). The Mongolian sheep pox virus, 2015 was closer to Tunisian and Chinese Gansu, Shanxi province strains. Chinese vaccine strain AV41, sequenced in this study was clustered with EF522181.1 Chinese Goat pox vaccine strain but Russian sheep pox vaccine strain, sequenced in this study was close to Mongolian goat pox viruses, 2009. The present data provides theoretical references to improve the preventive and control strategy. Based on the phylogenetic tree that we made, we conclude that SPPV and GTPV sequences in Mongolia were closer to Chinese SPPV, GTPV sequences therefore they were most likely imported from China.

Keywords: Goatpox, P32 gene, Phylogenetic analysis, Sheep pox, Vaccine strain

1 Introduction

Sheep pox, goat pox, and lumpy skin disease are caused by sheep pox virus (SPPV or Variola ovina) and goat pox virus (GTPV or Variola caprina), lumpy skin disease virus (LSDV), members of Capripoxvirus genus, Poxviridae family. SPPV is the largest virus in viruses, approximately 150 kbp and 96% and 97% nucleotides antigenically identical with goat pox virus and lumpy skin disease virus, respectively and cannot be distinguished by conventional serological test, pathogenesis, and clinical symptoms.

Lumpy skin disease is currently found only in African countries and Middle East [4,15]. In contrast, sheep and goat pox, endemic viral diseases are mainly prevalent to Asian, African developing countries. These diseases are classified as reportable animal diseases by the World Organization for Animal Health (OIE) in the last 50 years [1,3,13,16], but extended to Bangladesh in 1984 [14] India [6,17], and more recently to Yemen, Vietnam [8], and Mongolia [5]. In the past 43 years, since 1977 Mongolia has suffered from outbreaks of sheep pox (2006 – 2007, 2009, 2013, 2015, 2017) and goat pox (2008, 2009). Therefore, SPPV, GTPV are classified as a re-emerging disease in Mongolia. The SPPV and GTPV cause systemic diseases characterized by the fever, skin nodules, respiratory and gastrointestinal tract lesions, and lymph node enlargement in all ages of sheep and goats but these symptoms are most severe in young animals. Capripoxviruses are enveloped with a complex symmetry, double-stranded DNA viruses [22]. P32 is highly conserved structural protein shared by all Capripoxviruses. P32 contains major antigenic determinants [2,10], which are important for the pathogenicity, diagnosis, prevention, and control of capripoxvirus infections. Therefore, through sequence analysis, P32 gene is used to differentiate SPPV, GTPV and LSDV in between them. [9, 12, 15]. P32 protein is homologous to P35 that is encoded by Vaccinia virus H3L gene, and locates on the membrane surface of the mature intracellular viral particle [21]. Here, we sequenced P32 gene of two SPPV and two GTPV isolates, Perego M strain and a Russian vaccine strain of SPPV and a Chinese GTPV vaccine strain (AV-41). The sequences were used for the phylogenetic analysis by comparing them with various capripoxvirus isolates retrieved from GenBank to elucidate the genetic relatedness of these viruses.

2 Materials and Methods

Samples

For virus gene detection, seven samples were used. They were skin samples of crusted scab lesions from sheep and goats suspected with sheeppox and goatpox (SPPV-2017, SPPV-2015, GTPV-2009a, and GTPV-2009b), culture fluids of Russian and Chinese live vaccine strains, and vaccine fluid of the Perego M strain (PEREGO) originally from Morocco. Tissue samples of SPPV-2015 and SPPV-2017 were collected from diseased sheep in Sukhbaatar province, Mongolia in 2015 and 2017. Tissue samples of GTPV-2009a and GTPV-2009bwere collected from diseased goats in 2009 in Dornod Province, Mongolia. Russian and Chinese vaccine strains were inoculated in VERO cell culture and DNAs were extracted from the culture fluids. For Perego M strain, which is produced in Biocombinate SOI of Mongolia, DNA was extracted from the vaccine fluid.

DNA extraction

Total DNA was extracted from up to 25 mg tissue samples as well as live attenuated SPPV as positive control using the Instant virus DNA Kit (AJ Roboscreen, Germany). After complete lysis of the specimens by TLS Lysis solution and proteinase K, absolute ethanol was added, then the mixture was transferred to a spin column according to manufacturer`s protocol. Purified DNAs were recovered in 60 µl Elution buffer and applied to the Whatman FTA Elute Micro Card (GE Healthcare UK Ltd., Buckinghamshire, UK). DNA was extracted in Japan from a ϕ 3 mm of punched out card. The card was vortexed in 500 µl of sterile water for three times for washing. After removal of water, the sample was heated to 95°C for 30 min. in 30 µl sterile water for elution of DNA that was used for PCR.

Amplification of P32 gene by Polymerase Chain Reaction (PCR)

A nested polymerase chain reaction was performed to amplify the P32 gene using the DNA polymerase KOD FX Neo (TOYOBO, Osaka, Japan) and our designed primers (Supplementary Table 1). The primers were used at a final concentration of 0.3 µM. First-round PCR was performed as follows: initial denaturation at 94°C for 2 min; 35 cycles of 98°C for 10 sec, 50°C for 30 sec and 68°C for 90 sec; and final extension at 68°C for 7 min. The expected amplicon size was 1,523 base pairs (bp) based on a SPPV (AY077832). Second-round PCR was performed using the same PCR cycle parameters except for the primer annealing temperature at 56°C. The resultant fragment (1,370 bp) contains the full open reading frame (ORF, 972 bp in AY077832).

The PCR products were electrophoresed on a 1% agarose gel and amplified DNA fragments were retrieved using the Wizard® SV Gel and PCR Clean-Up System (Promega). Sanger sequencing of the fragments were performed by GENEWIZ Japan (Saitama, Japan). The obtained P32 gene sequences were already registered in the National Center for Biotechnology Information GenBank database.

Phylogenetic analysis

Sequence comparison of Mongolian SPPV and GTPV to the other available *Capripoxvirus* sequences in the Genbank database was performed using the online BLAST program. Sequence identities of nucleotides were analyzed using MUSCLE algorithm. A phylogenetic tree derived from nucleotide sequences was constructed for the *Capripoxvirus* using the neighbor joining method of MEGA (version X) software.

Vaccines

The sheep pox vaccine strain, which was an attenuated live Perego M strain and grown in primary lamb testis cell cultures, was produced at Biocombinat SOI, Mongolia. A Chinese goatpox vaccine strain AV41 is a live attenuated vaccine strain and was made in Jinyu Baoling biological manufacture, in China. A Russian vaccine strain was made in "ARRIAH" in Russia.

3 Results

Tissue DNA samples were extracted and amplified by a capripoxvirus specific PCR [18, 19, 20] at the Institute of Veterinary Medicine, Mongolia. A total of four field samples and three vaccine strains were tested and all were positive by nested PCR that we developed. The expected amplicon size for 1st and 2nd PCR samples are 1,523 bp and 1,370 bp, respectively. The p32 gene sequences of two SPPV field strains (Sukhbaatar, Mongolia 2015 and Sukhbaatar, Mongolia 2017), two GTPV field strains (Selenge, Mongolia 2009 and Dornogobi, Mongolia 2013), the Perego strain, a Chinese goat pox vaccine strain, and a Russian sheep pox vaccine strain were analyzed (Fig. 2) Total of 7 PCR products were sent for sequencing, and 7/7 nucleotide sequences of SPPV, GTPV P32 genes were obtained. BioEdit software was used for multiple alignment and phylogeny tree construction. Total of 3/7 sequences were belonged to Sheep pox virus, while 4/7 sequences were belonged to goat pox virus based on the phylogenetic tree results. Phylogenetic tree constructed by the neighbour-joining method based on the full sequences of P32 gene for 7 samples using a sequence of lumpy skin disease virus with accession number as AF124516 as an outgroup. The result showed that the two SPPV field strains of Mongolia were 98.05% identical, and other two GTPV field strains that originated from two different regions of Mongolia were completely identical (Fig. 1). The SPPV and GTPV field strains that I used in this study differed from each other (97% identity). Mongolian SPPV 2017 clustered together with Perego M strain and the Mongolian sheep pox virus 2015. A Chinese vaccine strain was clustered with Chinese GTPV vaccine strain (EF522181). A Russian SPPV vaccine strain was closer to a Mongolian GTPV 2009. Mongolian SPPV 2015 was closer to a Tunisian and a Chinese Gansu, a Shanxi province strains. They were clustered on a same SPPV clade. GTPV/Mongolia/2009 and Sheep pox/Russian vaccine strain belonged to the GTPV lineage. A goat pox vaccine strain was closely related to another Chinese goat pox vaccine strain (EF522181). GTPV/HuB/2009/China and GPV-ChongQ/2009 were clustered with GTPVs from Vietnam (Fig. 1).

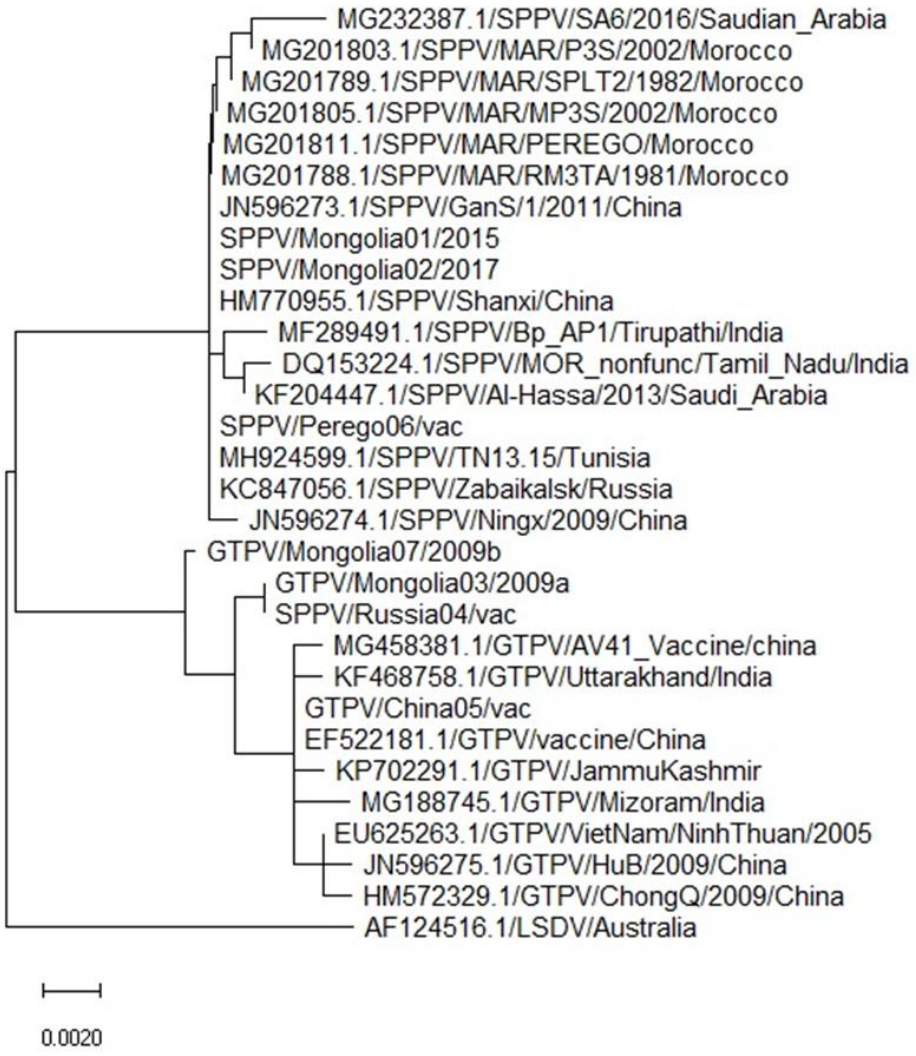

Fig. 1. Phylogenetic tree of capripoxviruses based on nucleotide sequence of P32 gene. Total of 7 PCR products were sent for sequencing, and 7/7 nucleotide sequences of SPPV, GTPV P32 genes were obtained. BioEdit software was used for multiple alignment and phylogeny tree construction. Total of 3/7 sequences were belonged to Sheep pox virus, while 4/7 sequences were belonged to goat pox virus based on the phylogenetic tree results. Phylogenetic tree constructed by the neighbour-joining method based on the full sequences of P32 gene for 7 samples using a sequence of lumpy skin disease virus with accession number as AF124516 as an outgroup. Bar shows 0.0020 nucleotide substitutions per site.

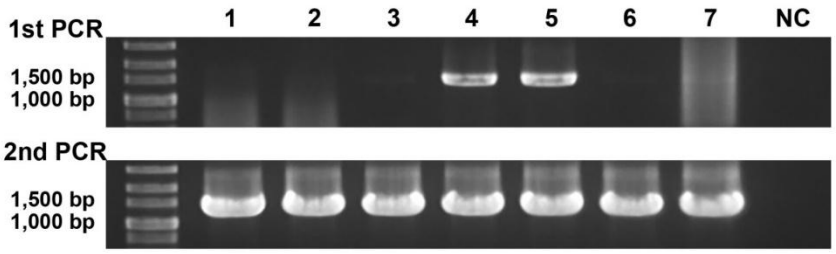

Fig. 2. Nested PCR result of the sheep and goat pox samples. Upper and lower panels show electrophoresis results in an agarose gel of 1st and 2nd PCR samples, respectively. The expected amplicon size in a sheep pox strain (AY077832) are 1,523 bp and 1,370 bp, respectively. The 2nd PCR products were sequenced. 1: SPPV-2015. 2: SPPV-2017. 3: GTPV-2009a. 4: SPPV Russian vaccine strain. 5: GTPV Chinese vaccine strain. 6: SPPV Perego strain. 7: GTPV-2009b. NC: Negative control.

Supplementary table 1. Primers that are used in the Nested PCR.

Name of primers	Sequence of primers
P32-F1 (forward)	ATCATGTACAGAGTAAAGTA
P32-R1 (reverse)	GTTATCAATGGGAAGAGATG
P32-F2 (forward)	TTCCGTTACCACTTGCTTCC
P32-R2 (reverse)	GGTGATGGATGGATGGTACA

4 Discussion

It is difficult to identify the source of the SPPV and GTPV which are currently prevalent in Mongolia. However, since SPPV and GTPV with similar genes have been detected in China, it is suspected that the source of the viruses might be imported from China. The natural outbreaks of capripoxvirus occurred in Mongolia between 2009 and 2017. The most remarkable feature of these outbreaks was their species specificity. Despite communal herding of sheep and goats the two endemic infections of Capripoxvirus in 2010 – 2017 caused clinical disease in sheep only, while the other outbreak in 2009 caused clinical disease only in goats.

Molecular characterization of the P32 gene supported this hypothesis of species specificity, with the SPPV and GTPV field strain showing differences between each other and close similarity to previously published sequences of SPPV and GTPV, respectively. This indicates that there were two incursions into Mongolia of Capripoxviruses in recent years, a GTPV field strain 2009 and a SPPV field strain 2015-2017 (Fig. 1).

It is not being possible to identify the source of specific incursion, although the Mongolian SPPV 2015 P32 gene sequence was identical to the sequence of several SPPV isolates from China. In this analysis, Russian SPPV vaccines were classified as GTPV. Since GTPV can infect not only goat but also sheep [7], the origin of this virus might have been GTPV. Although GTPV-2009a and GTPV-2009b were isolated in 2009 in the same region (Dornod Province), the genes were different. On the other hand, SPPV-2015 and SPPV-2017, which isolated in the same region (Sukhbaatar province) but in different years, they had the same P32 genes. These phenomena indicate that multiple SPPV and GTPV originated from different areas are distributing in Mongolia, and some of them continue to survive in the contaminated areas. However, since poxviruses have a common antigen among strains and cellular immunity shows a strong protective effect on poxvirus infection, the diseases would be controlled by developing appropriate vaccines.

5 Conclusion

The present study expands the data for spread trend research of Mongolian SPPVs and GTPVs and provides theoretical references to improve the prevention and control strategy. The outbreaks of SPPV, GTPV occurred in geographically distant areas of Mongolia were highly species-specific. Based on the phylogenetic tree that we made, we conclude that SPPV and GTPV sequences in Mongolia were closer to Chinese SPPV, GTPV sequences therefore they were most likely imported from China.

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Conflict of interest

The authors declare that they have no competing interests.

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