Detection and phylogenetic analysis of local capripoxvirus from necropsy specimens of sheep suspected of sheeppox infection

Opsporing en fylogenetische analyse van lokale capripokkenvirussen uit pathologische stalen afkomstig van schapen verdacht van een schapenpokkeninfectie

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ABSTRACT

Capripoxvirus infections including sheeppox virus (SPPV), goatpox virus (GTPV) and lumpy skin diseases virus (LSDV), which are listed by the OIE, are malignant, severe and highly contagious diseases in sheep, goats and cattle. This study reports the identification of a capripoxvirus in necropsy specimens, including skin and lung lesions, lymph nodes, spleen and cotyledon obtained from dead sheep suspected of sheeppox infection. PCR was used to compare its genome with GPPV and SPPV isolates using sequence analysis. PCR demonstrated that all necropsy specimens were positive and nucleotide sequencing of PCR products indicated a close relationship to SPPV.

SAMENVATTING

Virussen van het genus capripokken, zoals de zeer infectieuze schapenpokken (SPPV), geitenpokken (GTPV) en nodulaire dermatose (LSD), zijn opgenomen in de OIE-lijst omdat ze ernstige aandoeningen kunnen veroorzaken bij respectivelijk schapen, geiten en runderen. In deze studie wordt de identificatie beschreven van een capripokkenvirus uit letsels van de huid, long, lymfeknopen, milt en cotyledon van een gestorven schaap verdacht van een SPPV-infectie. In een PCR specifiek voor capripokken werd aangetoond dat alle stalen positief waren. Via een sequentieanalyse van de PCR-producten werd de sequentie van het geïsoleerde virus vergeleken met deze van gekende isolaten. Daaruit bleek een nauwe verwantschap met SPPV.

INTRODUCTION

The *Capripoxvirus* genus, a member of the *Chordopoxvirinae* subfamily of the *Poxviridae* family, includes sheeppoxvirus (SPPV), goatpox virus (GTPV) and lumpy skin disease virus (LSDV), all of which cause malignant, severe, and highly contagious diseases listed by the World Organisation for Animal Health (OIE) for sheep, goats and cattle, respectively (World Organisation for Animal Health, 2004; Mangana-Vougiouka *et al.*, 1999; Mangana-Vougiouka *et al.*, 2000; Tulman *et al.*, 2002 ; Gubser *et al.*, 2004; Hosamani *et al.*, 2004).

SPPV and GTPV infections are endemic and have been reported in different parts of the world including Africa, Nepal, Bangladesh, Iran, Afghanistan, India (Ramprabhu *et al.*, 2002; Parthiban *et al.*, 2005), the Middle East and Turkey (Gulbahar *et al.*, 2000; Oguzoglu et al 2006). The occasional introductions of SPPV and GPPV in Greece indicate the danger of spread to the European Community. The diseases cause severe financial loss in the international trade in animals and animal products due to mortality, abortion, reduced productivity, and lower quality of wool and leather (Markoulatos *et al.*, 2000; Mangana-Vougiouka *et al.*, 2000; Gulbahar *et al.*, 2000; Hosamani *et al.*, 2004; Parthiban *et al.*, 2005; Oguzoglu *et al.*, 2006).

Current diagnostic methods in laboratory diagnosis of the diseases are based on virus isolation in cell culture, electron microscopy and conventional serological methods, including virus neutralization (VN), immunofluorescence (IF) and enzyme-linked immunosorbent assay (ELISA) (Oguzoglu *et al.*, 2006). However, polymerase chain reaction (PCR) is the most reliable, sensitive, rapid and specific method, especially in terms of separating capripoxvirus from parapoxvirus infections (Ireland and Binepal., 1998; Markoulatos *et al.*, 2000, Oguzoglu *et al.*, 2006).

In this study, using PCR technology the identification of a SPPV is described from skin and organ lesions (lung, lymph nodes, spleen and cotyledon) collected during necropsy of a dead sheep suspected of SPPV infection. Phylogenetic analysis based on sequencing of the PCR products was also performed using genome sequence data from capripoxvirus isolates from all over the world.

Table 1. Goatpox and sheeppox virus isolates used for sequence comparison.

Nr	Virus	Strain	Country	Accession Number
1	Goatpox	Samsun/POX-TR/2006. (Local Isolate)	Turkey	DQ440578
2	Goatpox	Peller	Kazakhstan	AY077835
3	Goatpox	G20-LKV	Kazakhstan	AY077836
4	Goatpox	Bareilly/00	India	AY588599
5	Goatpox	Liujiang/2003	China	AY773088
6	Goatpox	Mukteswar	India	AY159333
7	Goatpox	P32 /mRNA	China	AY881707
8	Goatpox	Sampalbur/82	India	AY588601
9	LSD	Neethling Warmbaths	South Africa	AF409137
10	LSD	Neethling NI-2490	USA	AF325528
11	LSD	Neethling	South Africa	AF336131
12	Sheeppox	NSI	West Africa	AF124517
13	Sheeppox	TU/VO2127(SPV-7U)	Turkey	AY077832
14	Sheeppox	NISKHI	Kazakhstan	AY077834
15	Sheeppox	Rumanian Fanar	Rumania	AY368684
16	Sheeppox	Bareilly/00	India	AY588604
17	Sheeppox	Pune 183/03	India	AY588603

* Our local isolate (Samsun/POX-TR/2006) compared to other sheeppox and goatpox virus isolates.

MATERIALS AND METHODS

Necropsy Specimens

Necropsy specimens including nodular and proliferative skin lesions (SL), lung lesions (LL), lymph nodes (LN), spleen (S) and cotyledon (COT) were collected from a dead sheep suspected of sheeppox. These samples were used for PCR amplification by using specific primers for the gene encoding for the Capripoxvirus attachment protein.

Viral DNA extraction

The viral DNA of necropsy samples was extracted using the alkaline phenol-chloroform-isoamyl-alcohol (24:1) technique (Sambrock *et al.*, 1989). The samples were stored at -80 ºC until used as templates for PCR.

In the PCR for capripoxvirus, 10 µL of template were placed in 50 µL of the final volume of a 10 X reaction mixture containing 50 mM KCL, 10 mM Tris-HCl, 1.5 mM MgCl2, 200 mM of dNTP and 100 pmol of primer sets (P1, 5' TTT CCT GAT TTT TCT TAC TAT- 3'; and P2, 5' AAA TTA TAT ACG TAA ATA AC 3') for the gene encoding for the viral attachment protein as described by Ireland and Binepal (1998) and 2U Taq-DNA polymerase (Fermentase, Cat Nr:EP0402, Lithuania). Amplification consisted of an initial denaturation step at 94 ºC for 4 min, which was followed by 35 cycles at 47 ºC for 1 min, 72 ºC for 1 min, 95 ºC for 45 sec and, finally, extension at 72 ºC for 10 min in a thermal cycler (Biometra T1, Gottingen, Germany). PCR products were visualized in 1% agarose gel containing ethidium bromide. The PCR for alpha tubulin was used as an internal control to ensure that the DNA extraction was done correctly.

DNA sequencing and phylogenetic analysis

The PCR products were sequenced with a DTSC sequencing kit (Beckman Coulter, USA) in a Beckman Coulter CEQ 8000 Genetic Analyzer after cleaning using a DNA Clean-Up kit (Sigma, USA). The nucleotide sequences obtained from the PCR products were aligned with known sequences from representatives of the Capripoxvirus genus and a sequence identity matrix was calculated. Meanwhile, phylogenetic analysis was done on a phylogram generated with the Treeview (Page, 1996) program after analysis with Neighborhood Joining Analysis (NJA) using CLC Combined Workbench (CLCbio, Denmark) after bootstrapping. Nucleotide sequence data from the local isolate was submitted to GenBank which also assigned an accession number. The SPPV, GPPV and LSDV strains used for nucleotide sequence comparisons are presented in Table 1.

RESULTS

The expected amplicon size of 192 base pairs (bp) was observed in all of the necropsy samples (Figure 1: lanes 2, 4, 6, 8, 10). As an internal control, a 595 bp fragment from the same necropsy material was monitored using primers for the alpha tubulin gene (Figure 1: lanes 1, 3, 5, 7, 9).

The sequencing of the products yielded 100-125 nucleotides on the sample basis and they were all identical. The sequence identity matrix showed 98 to 100 % homology between the local virus (isolate) and those reported from Kazakhstan (AY077835,

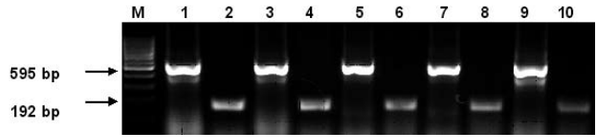

Figure 1. The results of polymerase chain reaction amplification of necropsy samples obtained from sheeppox virus suspected sheep. M: 100 bp DNA Ladder (Fermentas, Latvia). Lanes 2, 4, 6, 8 and 10: The DNA product of expected size 192 bp was detected in necropsy samples including skin lesion, lung lesion, lymph node, spleen and cotyledon, respectively. Lanes 1, 3, 5, 7 and 9: 595 bp fragments derived from PCR amplification of the same necropsy specimen using primers for alpha tubulin sequence for internal control.

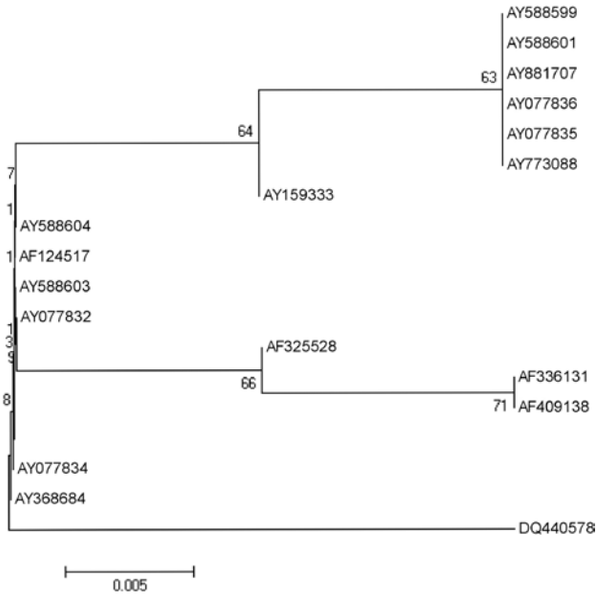

Figure 2. Phylogenetic analysis of the sheeppox virus isolated from necropsy specimens from sheep suspected of sheeppox infection. Phylogenetic analysis was done on a phylogram that was generated with the Treeview (Page, 1996) program after analysis with the CLC Combined Workbench (CLCbio, Denmark).

AY07736) and India (AY159333) (Table 1). The local isolate was also highly homologous with the Neethling Warmbaths (LW) strain (AF409138) of LSDV. The NJA showed that the isolate was more closely related to SPPV than GTPV (Figure 2).

Additionally, nucleotide analysis indicated that the virus isolated in this study was closely related to another virus (AY077832) which caused an outbreak of sheeppox in Turkey in the late 1970's. It was isolated by six passages in primary lamb testicle cells and one passage in sheep choroid plexus cells. It was subsequently re-isolated in 2000 from lung lesions in an experimentally infected sheep at the Plum Island Animal Disease Center, Greenport, NY, USA.

DISCUSSION

SPPV and GTPV infections in small ruminants are highly contagious. Laboratory diagnosis of the diseases is based on clinical signs and serological tests such as virus neutralisation, immunofluorescence, agar gel immunodiffusion (AGID) techniques, enzyme linked immunosorbent assay (ELISA), virus isolation or electron microscopy (Managana-Vougiouka *et al.*, 2000; Oguzoglu *et al.*, 2006). These techniques are time-consuming, difficult to apply routinely and pose an additional difficulty in terms of cross-reaction with the ORF virus (Mangana-Vougiouka *et al.*, 2000). In addition, SPPV and GTPV are extremely host specific (Rao and Bandyopadhyay, 2000) but in some countries both viruses can cross infect sheep and goats, which poses a problem in diagnosis and epidemiology. Although recent molecular studies suggest that the *Capripoxvirus* genus including SPPV, GTPV and LSDV are very similar in terms of antigenic characteristics, these viruses are phylogenetically distinct and can be differentiated by accurate molecular techniques (Bhanuprakash *et al.*, 2006). In this study, PCR revealed capripoxvirus specific 192 bp amplicons in all of the necropsy samples. Nucleotide sequence identity data obtained in this study indicates that the local isolate SAMSUN/POX_TR (GenBank Accession Nr: DQ440578) has nucleotide homology of 98% with Kazakhstan (AY077835), India (AY588599) and China (AY773088), 99% with India (AY159333) and 100% with Kazakhstan goatpox virus strains, respectively. Phylogenetic analysis revealed that the isolate was more closely related to SPPV strains (AY077832, AY368684, AY077834, AY588603, AY588604, AF124517 than to GTPV and LSD viruses. In addition, the analysis indicated substantial differences between the local attenuated vaccine virus strain (Bakirkoy strain) and the isolate. From this perspective, the use of attenuated live SPPV strains as a vaccine against SPPV and GTPVs may be of critical importance during enzootic and epidemic events. However, sheeppox disease that may be induced by the vaccine itself, vaccine errors and the use of live vaccines in non-endemic areas may initiate capripoxvirus ovis infection. The results of this study suggest that the isolated virus is distinct from the vaccine strain currently used in Turkey.

On the other hand, interspecies transfer of some critical viruses (e.g. peste des petits ruminants virus, pestiviruses and capripoxviruses) may occur and may be a consequence of mixed (sheep and goats) small-scale production units or uncontrolled animal movements between regions. Nevertheless, currently it is not possible to determine the likelihood of the latter speculation since the authors do not have access to regional capripoxvirus isolates. Previous studies reported that strains of SPPV and GTPV causing infection in both species were restricted to certain geographic regions, such as Africa, while Asian isolates were host-specific, as reported in India (Rao and Bandyodhyay, 2000; Tulman *et al.*, 2002; Bhanuprakash *et al.*, 2006). The sequencing and phylogenetic analysis results of this study identified our isolate as SPPV. When the nature of the small-scale farms of the region in which sheep and goats are housed together is taken into consideration, minor nuances in virus genomes will enable the poxviruses to gain the ability of infecting the other species in the region. However, even though the sequence identity matrix and phylogenetic analysis results indicate the isolate was SPPV, the data should be confirmed using specific primer sets for SPPV and GTPV.

In conclusion, from time to time SPPV outbreaks cause substantial financial losses in Turkey. Data obtained in this study indicate that phylogenetic examination of SPPV or GTPV isolates in Turkey could play an important role in developing an effective control program through epidemiological and eradication studies.

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