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Comparative sequence analysis of diagnostic PCR amplicons from Indian sheeppox virus

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ABSTRACT

Field samples were identified by agar gel precipitation and polymerase chain reaction (PCR) as positive for sheeppox virus. Envelope protein gene PCR amplicons (192 bp) of Indian sheeppox viruses isolated in 1997 and 2003 were sequenced to analyse nucleotide divergence. Analysis revealed that 2003 isolates possessed 100% nucleotide identity to each other, but only 95% nucleotide and amino acid were identity to an isolate from 1997. These Indian isolates were unique from other capripox virus sequences in that they contained a single codon insertion. Consistent with previous findings, the results indicate that recent sheepox virus isolates in India are more similar to previously described sheepox virus than to goatpox viruses from India and elsewhere.

Key words: sheeppox virus, India, nucleotide sequence, envelope protein, phylogenetic analysis

Introduction

The *Capripox* genus is currently comprised of sheeppox virus (SPV), goatpox virus (GPV) and lumpy skin disease of sheep, goat or cattle, respectively. Capripox virus infection of sheep, goat and cattle result in major economic losses in terms of mortality, reduced productivity and lower quality of wool and leather. Sheeppox and goat pox are endemic throughout southwest and Central Asia, the Indian subcontinent and Northern and Central Africa (CARN, 1993). There have been several reports of pox in sheep from different parts of India (SHARMA et al., 1986; RAO et al., 1996; MALIK et al., 1998).

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The genetic nature of SPV isolates would open new vistas in the field of epidemiology and effective control of this disease. Although methods such as polymerase chain reaction, followed by restriction endonuclease, analysis and hybridization methods can be used to study the genetic nature of virus, sequencing analysis is a very effective method to help understand the genomic nature of the virus. In the present study, nucleotide divergence of local sheeppox virus isolates were compared using sequence analysis of viral envelope protein gene.

Materials and methods

Sample collection and processing. A total of 22 scab samples were collected from two different outbreaks suspected of sheeppox in Tamil Nadu. One occurred in both sheep and goats in Hosur, Dharmapuri District of Tamil Nadu during 2001 and another during 2003 in the Kancheepuram District of this state among sheep. The scab materials collected separately from individual animals during the outbreaks were processed and prepared as 10% (w/v) suspension in sterile tissue culture medium (DMEM, Life Technologies, U.S.A.), clarified and stored at 4 °C until use.

Identification of etiological agent

1. Agar gel precipitation test. Agar gel precipitation test (AGPT) was conducted for confirmation of sheeppox and goat pox using soluble antigen (RAO and NEGI, 1997). The goat pox virus (Pirbani strain, India) was used as a positive control, and sheeppox hyperimmune serum raised against the Ranipet strain of sheeppox virus (vaccine strain) in rabbits were used.

2. Polymerase chain reaction (PCR). Polymerase chain reaction was carried out using the boiling method, without extracting DNA from infected scab material (IRELAND and BINEPAL, 1998). Around 20 µl of pox virus infected suspension was boiled for 10 minutes and used as a template for PCR. The reaction mix consisted of 5 x PCR buffer, 0.2 mM dNTP mix, 0.25 nM of forward and reverse primers, 2.5 units of Taq DNA polymerase (Medox Agencies, U.S.A.) and, finally, 5 µl of template was added to the reaction mix. The primer sequence and thermal cycle protocols described by IRELAND and BINEPAL (1998) were used in this study. The primer sequence of viral envelope protein was as follows:

P_1 5' TTTCCTGATTTTTCTTACTAT3'

P_2 5' AAATTATATACGTAAATAAC3'

The temperature profile consisted of an initial cycle of 95 °C for 5 min, 50 °C for 30 sec, 72 °C for 1 min, followed by 34 cycles of 94 °C for 1 min, 50 °C for 30 sec, 72 °C for 1 min, and a final elongation step at 72 °C for 5 minutes.

The amplified product was visualized in 1.5% agarose gel electrophoresis, together with a 100 bp ladder as a DNA marker. Of 22 samples tested, 14 were found positive

by AGPT and PCR. Among the 14 positive samples three representative samples were taken for virus isolation in primary lamb testis/lamb kidney cells. The three viral isolates, viz., SPV TN1 and SPV TN2, were from the 2001 outbreak at Hosur, and SPV TN3 was from the Kancheepuram outbreak during 2003. These three isolates were subjected to sequencing studies.

Sequencing of PCR products. The PCR products were purified using a silica membrane-based column purification kit (Life Technologies, U.S.A.). The purified product was checked in 1.5% agar gel electrophoresis. The multiple purified PCR products of SPV TN1, SPV TN2 and SPV TN3 isolates were then subjected to automatic sequencing by a commercial company (Bangalore Genei Pvt. Ltd, India) at both forward and reverse directions. Homology searches against all the published capripox virus sequences were carried out using BlastN and fastA programme (National Centre for Biotechnology Information, U.S.A.). The nucleotide sequence data of local SPV isolates were submitted to GenBank, who also assigned the accession number. The sheep and goat pox virus strains used for nucleotide sequence comparison are presented in Table 1.

Table 1. Sheep and goat pox virus isolates used for sequence comparison

S.N^o	Virus	Strain	Country	Accession Number
1	Sheeppox	SPV TN1	India	AY331145
2	Sheeppox	SPV TN2	India	AY331146
3	Sheeppox	SPV TN3	India	AY331147
4	Sheeppox	Rumanian	Rumania	AY368684
5	Sheeppox	Nigerian	Nigeria	AF124577
6	Sheeppox	SPV Niski	Kazakshtan	AY077834
7	Sheeppox	SPV A	Kazakshtan	AY077833
8	Goatpox	TU02127	Turkey	AY077832
9	Goatpox	G20-LKV	Kazakshtan	AY077836
10	Goatpox	Pellor	Kazakshtan	AY077835
11	Goatpox	Muktheswar	India	AY159338
12	Goatpox	Uttarkashi	India	AY382869

Results

The viral attachment protein primers used in PCR amplification produced the expected amplicon size of 192 bp. No PCR product was observed from uninfected control. The multiple nucleotide sequence alignment of SPV local isolates (SPV TN1, SPV TN2 and

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                          111 111 111 122 222 222 223 333 333 333 444 444 444
                  123 456 789 012 345 678 901 234 567 890 123 456 789 012 345 678
SPV NISKHI        GGT TTC ATT TTT TGG TAT ATT TGA TAT TAG TAT AAT AGG AGC ACT TAT
SPV 1             ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
SPV TU02127       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
SPV Nigerian      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
GPV Muktheswar    ... ... ... ... ... ... ... ... ... ... ... ... G.. ... ... ...
GPV G20LKV        ... ... ... ... ... ... ... ... ... ... ... ... G.. ... ... ...
GPV Pellor        ... ... ... ... ... ... ... ... ... ... ... ... G.. ... ... ...
SPV TN1&TN2       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
SPV TN3           .-. ... ... ... A.. ... ... ... ... ... ... ... ... ... ... ...
SPV Rumanian      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
GPV Uttarkashi    ... ... ..C ... ... ... ... ... ... ... ... ... G.. ... ... ...

                  455 555 555 556 666 666 666 777 777 777 788 888 888 889 999 999
                  901 234 567 890 123 456 789 012 345 678 901 234 567 890 123 456
SPV NISKHI        TAT TTT ATT TAT TAT AAT AAT GAT AAT TTT TAA TTT GAA TTC --- TAA
SPV 1             ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
SPV TU02127       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
SPV Nigerian      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
GPV Muktheswar    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
GPV G20LKV        ... ... ... ... ... ... ... ... ... ... ... ... A.. ... ... ...
GPV Pellor        ... ... ... ... ... ... ... ... ... ... ... ... A.. ... ... ...
SPV TN1&TN2       ... ... ... ... ... ... ... ... ... ... ... ... ... ... AAA ...
SPV TN3           ... ... ... ... ... ... ... ... ... ... ... ..G ... ..G ... AAA ...
SPV Rumanian      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
GPV Uttarkashi    ... ... ... ... ... ... ... ... ... ... ... ... A.. ... ... ...

                      111 111 111 111 111 111 111 111 111 111
                  999 000 000 000 011 111 111 112 222 222 222
                  789 012 345 678 901 234 567 890 123 456 789
SPV NISKHI        ATT ACT ATG GTT TTT AGC AGG TAT GTT ATT TAC
SPV 1             ... ... ... ... ..T ... ... ... ... ... ...
SPV TU02127       ... ... ... ... ..T ... ... ... ... ... ...
SPV Nigerian      ... ... ... ... ..T ... ... ... ... ... ...
GPV Muktheswar    ... ... ... ... ..T ... ... ... ... ... ...
GPV G20LKV        ... ... ... ... ... ... ... ... ... ... ...
GPV Pellor        ... ... ... ... ..T ... ... ... ... ... ...
SPV TN1&TN2       ... ... ... ... ..T ... ... ... ... ... ...
SPV TN3           ... ... ... ... ..T ... ... ... ... ... .T.
SPV Rumanian      ... ... ... ... ..T ... ... ... ... ... ...
GPV Uttarkashi    ... ... ... ... ..T ... ... ... ... ... ...
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Fig. 1. Multiple nucleotide seuqence analysis of sheep and goat poxvirus envelope protein gene

SPV TN3) with other sheep and goat pox virus isolates was carried out using MEGA software package (Fig.1.). The nucleotide sequence of local isolates were translated into corresponding amino acids, and compared. The percentage of nucleotide homology of local SPV isolates against other sheep and goat pox strains was calculated. It was found that SPV TN1 and SPV TN2 had more than 95% homology and that SPV TN3 had more than 90% homology, when compared to other sheep and goat pox viral isolates sequences available in the genbank. The phylogenetic analysis of sheep and goat pox virus isolates was carried out using Clustalx 1.8 and Phylip software packages.

Discussion

Few studies exist of the molecular biology of sheep and goat pox. Restriction analysis of genomes of the virus isolated from different countries revealed that sheeppox viruses are closely related (BLACK, 1986). In the present study, the viral envelope protein gene of recent and past SPV local isolates were amplified by polymerase chain reaction, sequenced to analyze the nucleotide divergence within SPV isolates.

Different sheeppox virus strains could not be differentiated by analysis of their proteins and serological methods (DAVIES and OTEMA, 1981). PCR-based diagnostic methods were effective in diagnosis of goat pox and sheeppox in suspected biopsy samples in the field (RAO and BANDYOPADHYAY, 2000). However, strain variation within sheeppox is not feasible based on the PCR technique. Hence, recently restriction endonucleoase and cross-hybridization were recently carried out to differentiate the strains of SPV (BLACK et al., 1996; GERSHON and BLACK, 1988) and nucleotide sequence analysis of SPV (TULMAN et al., 2002) was employed to distinguish one from the other. The sequencing of part of the viral envelope protein gene revealed that both past SPV isolates possessed 100% homology at nucleotide level. However, the recent and past SPV isolates possessed 96% homology both at nucleotide and amino acid levels. The past SPV isolates possessed 97% homology against other SPV strains and 95% homology against other GPV strains at nucleotide level. The recent SPV isolate possessed 94% homology against other SPV strains and 92% homology against other GPV strains at nucleotide level. These results clearly indicate that nucleotide sequence analysis could be used effectively to differentiate sheep poxvirus isolates. In comparison with other sheep and goat pox isolates our local sheep and goat pox isolates possessed a unique triplet nucleotide, i.e. AAA at the position of 91-93 in the viral attachment protein gene. However, more isolates need to be sequenced to confirm that this unique difference exists in all the local isolates. Very few amino acid changes were noticed between local sheep poxvirus isolates and other sheep and goat poxvirus strains. Only one amino acid change occurred between recent and past local isolates.

On phylogenetic analysis the recent local isolate was very much closer to Nigerian and Kazakshtan strains of sheep poxvirus than the past local isolates. The latter were very

much closer to the Romanian strain of sheep poxvirus which is currently used as one of the vaccine strains in India. However, all local sheep poxviruses were very different from goat poxviruses of India and other countries.

Live attenuated SPV have been used in enzootic and outbreak areas as a vaccine against sheeppox and goat pox (KITCHING et al., 1987a; KITCHING et al., 1987b). However, vaccine-induced disease, vaccine failure and use of live vaccine in non-endemic areas create the need for improving the existing vaccine against capripox (RAO and BANDYOPADHYAY, 2000) in India. The past SPV isolates slightly differ from the recent isolate both at nucleotide and amino acid levels of viral envelope protein gene, which is a major immunogenic portion of the virus (HEINE et al., 1999). The amino acid changes in the immunogenic proteins may alter the immunogenic property of the virus.

Unfortunately, vaccine strain (Ranipet strain) of Tamil Nadu, India is not included in this study. Hence, nucleotide sequence comparison and antigenic and immunogenic properties of field and vaccine strain are to be analyzed to confirm any change in the selection of vaccine strain as previously envisaged (RAO and BANDYOPADHYAY, 2000).

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SAŽETAK

Virus ovčjih boginja dokazan je u terenskim uzorcima imunodifuzijom u gelu i lančanom reakcijom polimeraze (PCR). PCR umnošci gena za protein ovojnice (192 pb) indijskih izolata virusa ovčjih boginja izdvojenih tijekom 1997. i 2003. razlikovali su se po nukleotidnom slijedu. Izolati iz 2003. međusobno su bili identični u 100% nukleotida, dok je izolat iz 1997. bio njima podudaran samo u 95% nukleotida i aminokiselina. Ti indijski izolati bili su jedinstveni u odnosu na druge viruse boginja koza po jednom ugrađenom kodonu. U skladu s prijašnjim istraživanjima, nalazi ukazuju da su nedavni izolati virusa ovčjih boginja u Indiji sličniji ranije opisanim virusima ovčjih boginja nego virusima kozjih boginja iz Indije i drugdje.

Ključne riječi: virus ovčjih boginja, Indija, nukleotidni slijed, protein ovojnice, filogenetska analiza