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Research Article

Differentiation of Sheeppox and Goatpox Viruses by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism

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In the present study, the partial gene sequences of P32 protein, an immunogenic envelope protein of Capripoxviruses (CaPV), were analyzed to assess the genetic relationship among sheeppox and goatpox virus isolates, and restriction enzyme specific PCR-RFLP was developed to differentiate CaPV strains. A total of six goatpox virus (GTPV) and nine sheeppox virus (SPPV) isolates of Indian origin were included in the sequence analysis of the attachment gene. The sequence analysis revealed a high degree of sequence identity among all the Indian SPPV and GTPV isolates at both nucleotide and amino acid levels. Phylogenetic analysis showed three distinct clusters of SPPV, GTPV and Lumpy skin disease virus (LSDV) isolates. Further, multiple sequence alignment revealed a unique change at G120A in all GTPV isolates resulting in the formation of *Dra I* restriction site in lieu of *Eco*R I, which is present in SPPV isolates studied. This change was unique and exploited to develop restriction enzyme specific PCR-RFLP for detection and differentiation of SPPV and GTPV strains. The optimized PCR-RFLP was validated using a total of fourteen (n=14) cell culture isolates and twenty two (n=22) known clinical samples of CaPV. The Restriction Enzyme specific PCR-RFLP to differentiate both species will allow a rapid differential diagnosis during CaPV outbreaks particularly in mixed flocks of sheep and goats and could be an adjunct/supportive tool for complete gene or virus genome sequencing methods.

Sheeppox; Goatpox; Differential diagnosis; PCR-RFLP

Sheeppox and goatpox are diseases of sheep and goats caused by sheeppox virus (SPPV) and goatpox virus (GTPV), respectively. These two viruses are the members of the *Capripoxvirus* (CaPV) genus of the *Poxviridae* family and closely related to lumpy skin disease virus (LSDV) of cattle, the other member of the genus[22]. The genome of CaPV consists of linear dsDNA, is of 150–160 kbp nucleotides long and has termini with cross-linked hairpins. The genome has terminally redundant sequences at both the ends and these sequences have reiterated inverted terminal sequences which are repeated in tandem. Double-stranded DNA is covalently linked at both ends[6]. The genome codes for more than 200 proteins ranging from host range to immunodominant P32, cytokines and complement analogues.

Clinically, both the diseases exhibit fever and generalised pock lesions. They cause high morbidity, mortality and trade restriction of sheep and goats and their by-products and hence, are considered as

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economically important[1]. Capripox viruses (CaPVs) usually have no host preference as they infect both sheep and goats[19]. However, sheeppox and goatpox are considered as different entities in India[13] and recently, it was confirmed, in other countries as well[2].

Capripox are enzootic in Africa, particularly to the north and west of the Sahara, in the Middle East and Far East, and the Indian subcontinent. In India, outbreaks of the disease occur frequently incurring economic losses to the sheep and goat industries[6,7,17, 20, 23, 24]. Both the diseases have major impact on small ruminant productivity in countries where disease are enzootic, due to reduced milk yield, damage to hides and mortalities[26]. Mortality in young animals can exceed 50% and occasionally up to 100% in naive animals[6]. As the losses due to capripox in Maharashtra (India) state alone, with an average morbidity and mortality, are estimated over INR 105 million (US$2.3 million) and annual loss at the national level extrapolates to INR 1250 million (US$ 27.47 million)[8].

Control and eradication of any infectious disease rely on an effective vaccine and a suitable diagnostic tool for early detection of the causative agent. For capripox control in India, live attenuated vaccines are available. Despite the availability of several diagnostic tools for capripoxviruses[19], a simpler and more effective tool is always desirable. It is difficult to differentiate SPPV and GTPV based on the clinical picture as infection and pathogenesis of the diseases are alike. They are closely related to each other antigenically, which makes them indistinguishable serologically. It is now proved that cross infections of sheeppox and goatpox can occur[7,25]. The species of the virus is normally assumed to be defined by the host, but this is not always so and this approach needs to be replaced with molecular techniques for unequivocal differentiation of the species. It is also needed when low virulence strains cause similar infections in both sheep and goats, or some strains circulate in conflicting host species as reported earlier[13]. Recently, infection of goats by sheeppox virus has been confirmed using sequencing of the full length P32 gene in an experimental animal study[7] in India and also in Vietnam where some CaPV isolates shown distinct host preferences for goats rather than sheep[1]. So, it is essential that both species of capripoxvirus should be distinguished when both viruses affect both target species.

P32, one of the structural proteins conserved among all the capripoxviruses, contains major immunogenic determinants and has been targeted by many researchers to develop several molecular diagnostic tools for unequivocal detection and differentiation of CaPV[7,9,10,12,13,18,23]. It is analogous to P35 protein of vaccinia virus expressed on the envelope of the mature intracellular virion and may play a role in virus attachment, virulence and virus assembly.

Earlier, in our laboratory, duplex PCR targeting two different genes of CaPV genome for detection and differentiation of SPPV and GTPV isolates have been attempted[10]. However, differentiation of two viruses based on two different genes is not always reliable and is expensive. The final confirmation of either species from cell culture isolates or suspected clinical sample is done by commercial sequencing of the full length P32 gene of CaPV genome. As it involves more time and cost, it is not always employed. Therefore, differentiation by a simple PCR-RFLP targeting a conserved gene could be preferred to tedious cloning and sequencing. In this study, a restriction enzyme specific differentiation technique, in the form of PCR-RFLP on the attachment gene (partial gene of P32 protein) of CaPV using two different enzymes, is developed and evaluated using known CaPV isolates and clinical specimens from sheep and goats.

MATERIALS AND METHODS

Cells, viruses and clinical samples

The vaccine viruses, goatpox virus (Uttarkashi) at passage level 60 and sheeppox virus vaccine strains Srinagar, Ranipet and Rumanian Fanar (RF), were all propagated in Vero cells using EMEM with 2% bovine calf serum (BCS) and used for initial optimization of PCR-RFLP. Other GTPV and SPPV isolates/strains used in validation of the assay are shown in Table S1(Supplementary information). The clinical samples used in this study were either collected from field outbreaks or referred by the regional diagnostic laboratories to Pox Virus Laboratory, Division of Virology for confirmation. The viruses were harvested at 80% cytopathic effects (CPE) in Vero cells. Clinical samples homogenized as 10% suspension using phosphate buffer saline were used for extraction of total genomic DNA and stored at -20℃ until use.

Extraction of genomic DNA and PCR amplification

Total genomic DNA was extracted from harvested

infected cells and processed clinical specimens, using commercial DNA extraction kit as per manufacturer's protocol (AuPrep™, Life Technologies Pvt. Ltd., New Delhi, India). PCR was performed using reported primers[14] for amplification of attachment gene of CaPV genome using extracted gDNA with modifications. In brief, PCR reaction was carried out in a 50 μL volume containing specific primers for attachment gene and 2 μL extracted DNA using Jump Start™ Taq DNA polymerase system (Sigma, USA). The PCR thermal profile contained an initial denaturation at 95℃ for 5 min followed by 35 cycles of 94℃ for 30 s, 53℃ for 45 s and 72℃ for 1 min and final extension at 72℃ for 7 min. The PCR amplicons were analysed in 2% agarose gel containing ethidium bromide (EtBr) under UV transilluminator.

Cloning and sequence analysis of attachment gene

The attachment gene of GTPV (n=3) and SPPV (n=4) isolates were amplified using CaPV specific primers[14] and the purified amplicons were cloned into pGEMT-Easy vector (Promega, Madison, USA) as per standard procedures and sequenced in an automated DNA sequencer (ABI PRISM 3100, Perkin Elmer, California, USA). The edited sequences were submitted to NCBI GenBank (Accession No.GQ442629, GQ442630, GQ442632, FJ748487, GQ396154, GQ396155 and GQ396156) and compared with other published sequences. The details of sequences used in the study were shown in Table S2(Supplementary information). The sequences of attachment genes were assembled by using the MegAlign program of the DNASTAR package (Lasergene 6.0, DNASTAR Inc., USA). Phylogenetic tree was constructed based on deduced aa sequences by using MEGA 5.04 program[21]. From the sequence alignment report, unique restriction sites specific for each of the CaPV species were identified in order to develop RE specific PCR-RFLP for differentiation of CaPV strains as SPPV and GTPV.

Optimization of PCR-RFLP

Initially, restriction digestion of attachment gene amplicons from vaccine viruses of CaPV was optimized using two different enzymes namely *Eco*R I and *Dra* I (MBI Fermentas, Madison, USA) respectively for SPPV and GTPV isolates. Digestions of GTPV and SPPV using *Eco*R I and *Dra* I enzymes respectively were kept as negative controls. In brief, the reaction was carried out in a 25 μL volume containing 12.5 μL product and 10 U of enzyme at 37℃ for 2–3 h. The digested products were analysed in 2.5% agarose gel electrophoresis using EtBr stain under UV transilluminator.

Evaluation of PCR-RFLP

After optimization, the attachment gene based PCR-RFLP was applied to known cell culture adapted SPPV and GTPV isolates. Each reaction contained negative controls as mentioned earlier. All the isolates analysed by PCR-RFLP were confirmed by automated sequencing to check the specificity of the assay. After evaluation using cell adapted isolates, the PCR-RFLP was directly employed on known clinical samples of CaPV (n=22) to differentiate as GTPV or SPPV. The details of CaPV isolates and clinical samples used in validation of the technique were shown in Table S2(Supplementary information).

RESULTS

PCR, cloning and sequence analysis of attachment gene

The CaPV specific primers amplified approx.192 bp fragments (Fig. 1) as expected, when visualized in 2% agarose gel using ethidium bromide staining. All the GTPV and SPPV isolates as well as selected clinical samples produced this size of product. The amplified products from purified viral DNA of GTPV (Uttarkashi) and SPPV (Srinagar, Ranipet and RF strains) and other CaPV isolates yielded 191 bp sequences instead of the 192 bp expected, when cloned and sequenced commercially.

Comparison of edited sequences of SPPV and GTPV isolates with published sequences of attachment gene of CaPV strains/isolates including LSDV (Table 1) shows a high degree of identity except for some nucleotide substitutions. It showed identities of 99.4%–100% and 98.2% among GTPV field isolates and 100% among SPPV field isolates at both the nt

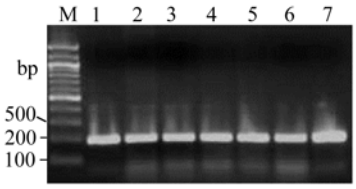

Fig. 1. Agarose gel electrophoresis (2% gel) showing amplification of attachment gene (approx. 192bp) of GTPV, Uttarkashi (Lane 1), GTPV, Ladakh (Lane 2), GTPV, Akola (Lane 3), SPPV, Srinagar (Lane 4), SPPV, Ranipet (Lane 5), SPPV-RF (Lane 6), SPPV-Pune/08 (Lane 7) and Lane M: 100 bp DNA ladder.

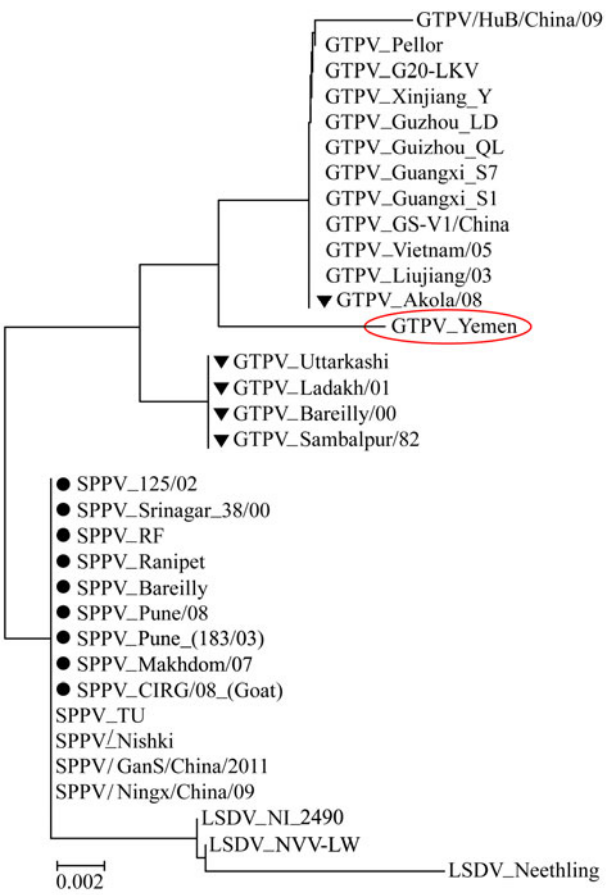

Fig. 2. Phylogenetic analysis of attachment gene sequence of Indian and other isolates SPPV and GTPV including LSDV showing three distinct clusters of members of the genus Capripox virus.

and aa levels. CaPV isolates gave 98.3%–98.8% and 96.5% homology between GTPV and SPPV at nt and aa levels. Phylogenetic analysis showed that the Indian GTPV and SPPV field isolates and vaccine viruses were distinctly separated different from other isolates (Fig. 2) and from lumpy skin disease virus (LSDV), the other member of the genus CaPV.

Identification of restriction sites

Multiple sequence alignment revealed unique two nt substitutions namely A81G and G129A in all the GTPV Indian isolates when compared to corresponding SPPV isolates. However, the G129A change was not found in the GTPV-Yemen isolate. Due to these changes, two restriction enzyme sites were formed, namely *Eco*R I (GAATTC) at 129–134 bp and *Dra* I (TTTAAA) at 126–131 bp respectively, for SPPV and GTPV isolates (Fig. 3), except GTPV-Yemen isolate.

Optimization of PCR-RFLP

When RE digestion of amplified attachment gene product of both viruses was performed using two enzymes, the digestion patterns for GTPV and SPPV strains differed. The vaccine virus of GTPV showed two fragments of 128 bp and 63 bp in size with *Dra* I enzyme, but none when *Eco*R I enzyme was used (Fig. 4A). Similarly, the vaccine viruses of SPPV produced two digested products using *Eco*R I enzyme of 129 bp and 62 bp but none with *Dra* I (Fig. 4B).

Evaluation of PCR-RFLP

A total of six GTPV and nine SPPV isolates produced a similar pattern of results as observed in vaccine viruses (Fig. 5). However, a notable exception was observed with two isolates from suspected goats which were originally thought to be of GTPV (CIRG/16/08 and 17/08) and were found to be SPPV,

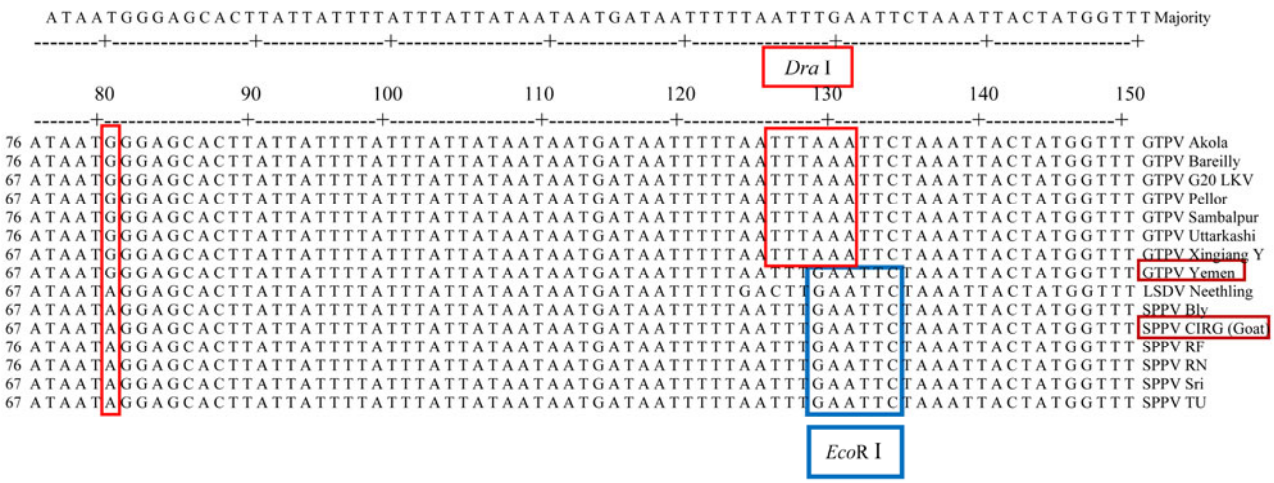

Fig. 3. Multiple sequence alignment of attachment gene sequences of CaPV isolates showing two distinct nt substitutions such as G81A and A129G (boxed) in SPPV isolates.

a result confirmed by full length sequencing of P32 gene, as reported earlier[7]. After validation of the technique using cell culture adapted CaPV isolates, it was employed on twenty two positive clinical samples all were found to identify the species exactly (Fig. 6). Only two clinical samples of goat origin (Sample No. 36 and 37 in Table 2) were found to be SPPV.

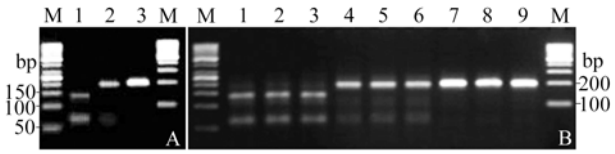

Fig. 4. Optimization of PCR-RFLP using purified genomic DNA of CaPV. A: GTPV (Uttarkashi, P60) showing clear digestion of amplicon in to two fragments by *Dra* I (Lane 1), digestion by *Eco*R I (Lane 2), undigested product as control (Lane 3) and Lane M: 50 and 100 bp markers (MBI, Fermentas, USA). B: SPPV (Srinagar, Ranipet and RF strains) showing digestion of all strains by *Eco*R I (Lane 1-3), digestion by *Dra* I (Lane 4-6) and undigested products as control (Lane 7–9).

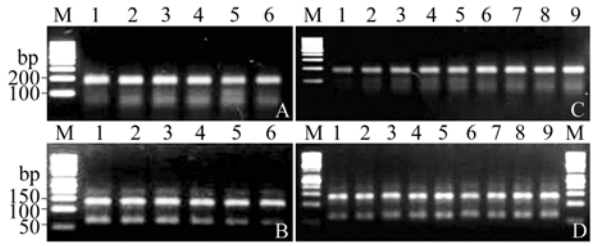

Fig. 5. Evaluation of PCR-RFLP method using CaPV field isolates. Agarose (2.5%) gel analysis showing: A: *Eco*R I specific digestion of GTPV isolates (Lane 1–6: Uttarkashi at P60, Uttarkashi at P30, Akola, Ladakh, Sambalpur and Avikanagar); B: *Dra* I digestion of above mentioned GTPV isolates. C: *Dra* I specific digestion of SPPV isolates (Lane 1–9: Srinagar-P40, Srinagar at P6, Ranipet, RF, Makhdoom, MKD-16 & MKD-17 (Goat origin), Pune/08 and Ahmadabad); D: *Eco*R I digestion of SPPV isolates.

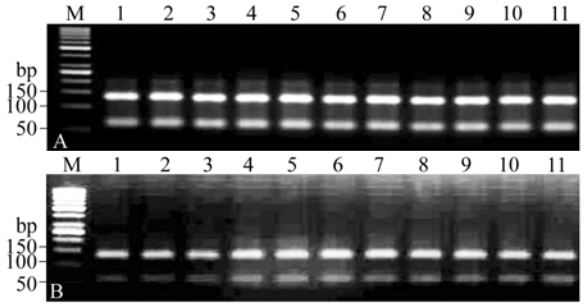

Fig. 6. Validation of PCR-RFLP method using known clinical samples. A: M, Marker; Lane 1-11: GTPV suspected samples digestion with *Dra* I. B: M, Marker; Lane 1-11: SPPV suspected samples digested with *Eco*R I showing clear differentiation of both species.

DISCUSSION

Sheeppox and goatpox cause similar pathogenesis and lesions in sheep and goats, but are considered to be two different entities worldwide[8]. Infection of GTPV in sheep and SPPV in goats is possible in a mixed flock of these species. Therefore, an explicit way of differentiation by some nucleic acid based technique is needed as they are not distinguishable by any serological assays due to close antigenic relationship[13,15]. Identification of CaPV strains by various serological assays had been in use for several years[19]. Due to low sensitivity and specificity, these tests have been replaced by nucleic acid based tools namely genomic RFLP[11], PCR based diagnostic assays viz. PCR/real time PCR/duplex PCR[3,4,10,12,14,16] and sequencing of some immunogenic/virulent genes[5,13].

The ultimate confirmatory diagnosis of any causative agent is done by sequencing a part or as a whole genome. But, it is time consuming, needs expertise and special reagents and is not a cost effective approach in a resource limited situation. Moreover, the RFLP based on whole genomic viral DNA is not efficient for distinguishing the very closely related SPPV and GTPV strains[10]. So, such a technique targeting a portion of a full gene would be rapid, economical and reliable in a common diagnostic facility. The attachment gene has already been used in the analysis of Indian SPPV isolates[18] and to check the antigenic relationship between LSDV and SPPV strains[9].

Earlier, SPPV and GTPV isolates of Indian origin were differentiated using PCR-RFLP targeting the whole P32 gene, which is of more than 1kbp in length using *Hin*f I enzyme. This produces three fragments for SPPV and two for GTPV as they have two and one *Hin*f I sites in their respective genes[13]. To reduce the time and cost involved, a similar approach targeting a part of P32 gene would be preferred for unequivocal differentiation of these CaPV strains. To do this, a simple PCR-RFLP targeting attachment gene was developed and evaluated using known CaPV isolates and clinical specimens, the results being further confirmed by *DNA polymerase* gene based PCR/real time PCR[3].

All the 22 clinical samples were detected by both attachment[14] and *DNA polymerase* gene based PCR[3] assays as CaPV. However, the PCR-RFLP using *Eco*R

I and *Dra* I enzymes could distinguish both species of CaPV by producing two fragments after digestion. It was found that *Eco*R I could digest only SPPV DNA but not the GTPV DNA and *Dra* I has the reverse effect (Fig. 6). On analysis of eleven isolates by sequencing, the PCR-RFLP is found to have 100% diagnostic sensitivity as compared to the commercial sequencing method. One nt substitution between GTPV and SPPV isolates at A129G was successfully employed to discriminate these species in a simple and cost effective manner. It enables timely vaccination using a specific antigen in a mixed flock of sheep and goats suspected to have infected by both viruses of CaPV. In recent past, association of sheeppox outbreak with GTPV[25] and goatpox with SPPV[6] has been proved by molecular techniques. In such conditions, a simple and cost effective differentiation of CaPV strains using PCR-RFLP would be a useful addition to other molecular techniques. However, the sequence and phylogenetic analysis of CaPV isolates revealed that GTPV-Yemen was not clustered with GTPV but SPPV. Thus if the developed PCR-RFLP tool was not able to identify CaPV as either SPPV or GTPV due to mutational changes or importation of some foreign strains to India, differentiation of these species should be done using full length P32 gene or sequencing of whole genome of the virus isolate to be studied. In this case the developed tool will still act as a preliminary screening technique for identification and a simple way of differentiation of CaPV isolates of Indian origin.

In conclusion, the present study demonstrated that a means is available to differentiate the closely related. SPPV and GTPV species using attachment gene based PCR-RFLP. This can be of effective and rapid (only 5 hr including PCR and RE digestion) compared to cloning and sequencing, which needs at least 3–5 days in a resource limited laboratory settings. After validating with large number of known clinical specimens, it could be a simple alternative to the high cost sequencing method of differentiating these strains.

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Supplementary information is linked to the online version of the paper on the Virologica Sinica website. www.virosin.org

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