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Evaluation of Duplex PCR and PCR-RFLP for Diagnosis of Sheep Pox and Goat Pox

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Abstract: In the present study, we evaluated the diagnostic efficacy of attachment gene based PCR (A-PCR) and A-PCR-RFLP employing a number of *capripoxvirus* field samples received from different parts of the country during 2000-2005. The tests were compared with duplex PCR and A-PCR-RFLP methods. We also cloned and sequenced A-PCR products from three representative Goatpoxvirus (GPV) isolates and three representatives Sheeppox Virus (SPV) isolates. F-PCR products of two isolates of GPV were also cloned and sequenced. Sequence variation in the attachment and fusion gene fragments of SPV and GPV was also determined to confirm the differential amplification in the suspected clinical samples. Phylognetic analysis based on partial fusion gene sequence of capripoxviruses showed that Indian isolates are closely related to other GPV strains published earlier.

Key words: Goat pox, sheep pox, diagnosis, PCR

INTRODUCTION

Goat pox and sheep pox, categorized under notifiable OIE (Office internationale des Epizooties) diseases are malignant pox diseases of goats and sheep, characterized by pyrexia and generalized pock lesions on skin and internal organs causing high mortality especially in the young ones. The diseases are caused by Goatpox Virus (GPV) and Sheeppox Virus (SPV) in goats and sheep, respectively. These two viruses, along with Lumpy Skin Disease Virus (LSDV) of cattle have been grouped in the *Capripoxvirus* genus of the *Poxviridae* family[1]. Goat pox and sheep pox are currently endemic in the Middle East, the Indian Subcontinent and Central and Northern Africa inflicting heavy economic loss.

Although the isolates of GPV and SPV display a host preference, clinical signs and pathogenesis of the disease are quite similar. The viruses are antigenically closely related and therefore, serologically indistinguishable[2]. The diseases are readily diagnosed in acute form by typical pock lesions on skin and lungs depending upon the species of animal involved. However, low virulence strains of capripoxvirus or orf (scabby mouth) can pose problems in differential diagnosis. Differentiation of SPV and GPV is essential particularly in an outbreak affecting both sheep and goats in a mixed flock. Capstick[3] and Kitching and Taylor[2] have also reported some strains of capripoxviruses to cause disease in more than one animal species. Capripoxvirus infection involving both sheep and goats in a mixed flock warrants the development of more reliable assay that differentiates GPV and SPV precisely. In this study, we report evaluation of capripox PCR for detection and differentiation of SPV and GPV by duplex PCR and PCR-RFLP. Sequence variation in the attachment and fusion gene amplicons of SPV and GPV was also analyzed to confirm the differential amplification in the suspected clinical samples.

MATERIALS AND METHODS

Viruses: Cell culture adapted capripoxviruses viz, Mukteswar 1946, Uttarkashi-1978 and Sambalpur-1984 (SA84) isolates of GPV[4-6] and Ranipet strain of SPV were taken from the virus repository maintained in poxvirus laboratory. The viruses were grown on preformed Vero cell monolayers and harvested when 90% of the cells showed cytopathic changes. The infected cell lysates were used for isolation of viral DNA.

Clinical samples: Skin scab materials collected from field outbreaks that occurred during last five years (2000-2005) in different parts of the country were included in this study. The tissue materials were ground to make a 10% (w/v) suspension in sterile distilled water and used in counter immunoelectrophoresis[7] for detection of capripoxvirus antigens and PCR. Both positive and negative control samples were also used in parallel in these tests.

Isolation of viral DNA from infected cells and scab materials: Viral DNA was isolated from Vero cells infected with GPV and SPV and also from scab tissues for

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PCR following the method using phenol chloroform method[8]. Briefly, 0.5 mL of infected cell lysate or 10% scab suspension was clarified by centrifugation at 1500 g for 10 min) and DNA was isolated by treatment with proteinase K (1.0 mg mL^{-1}) followed by extraction with phenol: chloroform: isoamyl alcohol (25:24:1). The DNA was precipitated with absolute ethanol after addition of 3M sodium acetate (pH5.2), washed with 70% ethanol and finally dissolved in 20 µL of water after air drying the pellet.

Amplification of attachment and fusion genes: The primers reported by Ireland and Binepal[9] were used for amplification of attachment and fusion genes of GPV and SPV. PCR amplification was carried out in 50µL reaction volume using primers specific for either attachment gene (A-PCR) or fusion gene (F-PCR) or both the primers together (AF-PCR). Attachment gene primers [forward primer-TCC GAG CTC TTT CCT GAT TTT TCT TAC TAT; Reverse primer-TAT GGT ACC TAA ATT ATA TAC GTA AAT AAC]; fusion gene primers [forward primer-ACT AGT GGA TCC ATG GAC AGA GCT TTA TCA; reverse primer-GCT GCA GGA ATT CTC ATA GTG TTG TAC TTC G]. PCR had initial denaturation step of 94°C for 4 min followed by 29 cycles of 94°C for 1 min, 53°C for 1 in, 72° C for 1 min and final extension of 72°C for 7 min. An aliquot (5 µL) of PCR product was analyzed by 1.0% agarose gel electrophoresis to visualize the PCR amplicons.

PCR-RFLP attachment gene: Restriction digestion of amplicons of attachment gene was done using EcoRI (MBI Fermentas, Madison, WI, USA). Briefly, 15 µL PCR product was digested with 10 units of EcoRI in a volume of 20 µL at 37°C for 3 hrs. The digested DNA was resolved in 1.5% agarose gel containing ethidium bromide at 70V for 2 hrs and the gel was visualized in a UV transilluminator.

Cloning and sequencing of amplicons of attachment and fusion genes: The PCR amplified attachment gene products from three representative isolates each of GPV (GPV–LAD01, GPV-SA84, GPV-BLY isolates) and SPV (SPV-183, SPV-125, SPV-BLY isolates) and fusion gene products from two isolates of GPV (GPV-LAD, GPV-SA84) were gel purified and cloned into pGEM-T Easy Vector (Promega Corporation Inc., Madison, Wisconsin, USA), following the manufacturer's protocol. The recombinant clones carrying attachment and fusion gene fragments were verified by colony PCR and restriction enzyme analysis. Sequencing was done using Automated DNA Sequencer (ABI Prism, model 377, version 3.0). The sequence obtained thus was aligned and analyzed to observe the homology with sequences available in NCBI database using MegAlign programme of Lasergene 6.0 (DNASTAR Inc, USA). Phylogenetic analysis was carried out using MEGA version 3.0[10].

RESULTS

PCR, duplex PCR and PCR-RFLP: A 191 bp fragment of attachment gene was amplified from scab samples and cultured cells infected with either SPV or GPV. AF-PCR resulted in amplification of only 191 bp fragment with ovine samples and two fragments of 472 and 191 bp in all the caprine samples (Fig. 2). A total of 49 capripox samples from caprine and ovine origin were screened (Table 1). Out of a total of 49 samples screened, 17 samples were positive in CIE while 27 samples were positive for either SPV or GPV by multiplex PCR. Out of 27 PCR positive samples, 18 were from ovine origin representing SPV samples, while the remaining 9 samples that gave two bands in the AF-PCR were from goat origin representing GPV samples. PCR-RFLP of A-PCR products from all the samples showed two bands of 129 bp and 62 bp in samples where as none of the amplicons from caprine samples got digested by EcoRI (Fig. 1).

Sequence analysis of attachment and fusion genes: Sequencing of the cloned PCR products of attachment gene of three isolates each of SPV (SPV-183, SPV-125, SPV-BLY) and GPV (GPV-LAD, GPV-SA84, GPV-BLY) and fusion gene of two GPV isolates (GPV-LAD, GPV-SA84) was carried out. The comparison of the sequences of the attachment gene of GPV, SPV and Lumpy Skin Disease Virus (LSDV) revealed few nucleotide substitutions (Fig. 3). Multiple alignments of attachment gene sequences of SPV and GPV isolates revealed two nucleotide substitutions including G81A and A129G in SPV. Similarly, comparison of fusion gene sequence of Indian isolates of GPV with published sequences of GPV isolates revealed two nucleotide substitutions i.e. T replaced by C at position 218 while T was replaced by A at 334 position in Indian isolates (data not shown). But comparison of fusion gene sequence of GPV isolates with SPV and LSDV indicated 16 nucleotide substitutions at different positions. The percent similarity of attachment

Table 1: Comparison of diagnostic efficacy of CIE, PCR and PCR-RFLP methods for detection of capripoxvirus

Samples	Scab	A-PCR	PCR-RFLP	F-PCR	Duplex PCR
11	Goat	9	-	9	9
38	Sheep	18	18	-	18
49		27	18	9	27

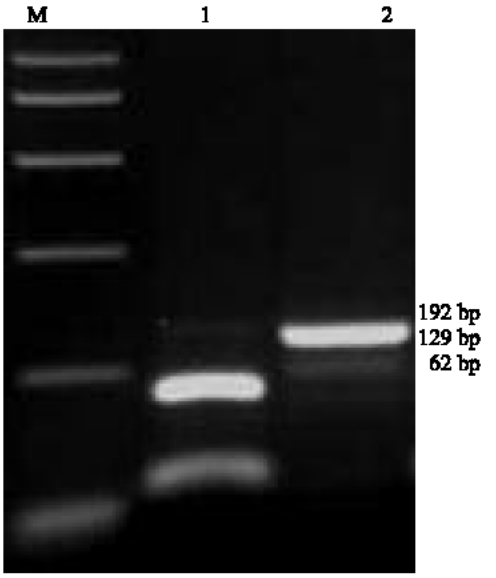

Fig. 1: PCR-RFLP method of differentiating SPV and GPV samples based on restriction digestion of attachment gene. M-PCR marker; Digestion of PCR amplicon derived from SPV using EcoRI enzyme yields two bands of 129 and 62 bp fragments (Lane 1) while amplicons derived from GPV samples are not digested

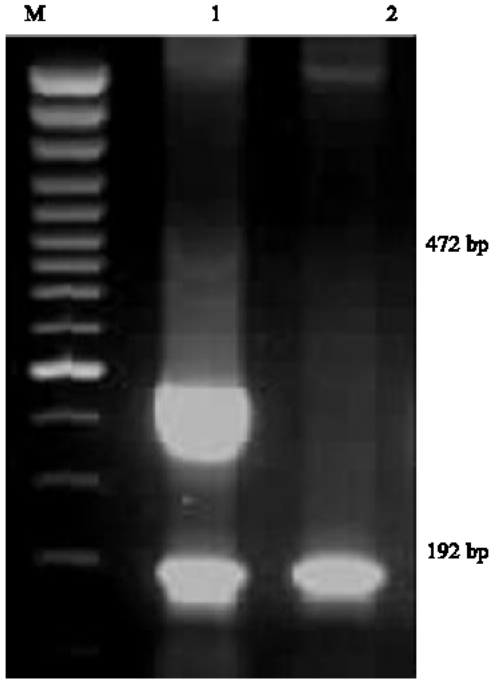

Fig. 2: Duplex PCR method for detection of differentiation of capripoxvirus. M-100 bp ladder; Two amplicons of 472 and 191 bp are obtained in case GPV samples representing fusion and attachment gene fragments respectively, while only 191 bp attachment gene fragments got amplified from SPV samples

gene with GPV and SPV was 98-100 whereas with LSDV it was 97%. The percent similarity based on fusion gene sequence was 93-95% between SPV and GPV, 97% between LSDV and GPV and 94% between LSDV and SPV. The phylogenetic analysis based on the partial fusion gene sequences of different capripoxviruses showed three distinct clusters (Fig. 4), with Indian isolates of GPV sub clustering with the previously published GPV sequences.

DISCUSSION

Although ELISA and virus isolation in cell culture are sensitive, they are tedious, time consuming and also they fail to detect virus particles that are bound to neutralizing antibody[9]. The Restriction Fragment Length Polymorphism (RFLP) of entire viral DNA could barely distinguish GPV and SPV[11]. Of late, polymerase chain reaction (PCR) assays targeting attachment and fusion gene sequences of capripox viruses have been reported for the detection of capripox viruses in skin biopsies and infected cell culture fluids[9,12,13]. In the present study, we evaluated the diagnostic efficacy of attachment gene based PCR (A-PCR) and A-PCR-RFLP employing a number of capripoxvirus field samples received from different parts of the country during 2000-2005. Further in extending this work, we performed the fusion gene PCR for capripox samples of ovine origin. Based on the results of both A-PCR and F-PCR, we also devised duplex PCR for detection and confirmation of sheeppox and goat pox suspected samples in a single tube reaction.

Results of A-PCR and F-PCR were compared with CIE, a preliminary screening test for detection of capripoxvirus antigen employed in our laboratory. Capripox specific PCR, A-PCR showed higher (55%) positivity in comparison to 37% by CIE test (Table 1), reflecting poor sensitivity of the latter. Lack of amplification of the 18 samples that originated from sheep by F-PCR clearly showed that the test failed to amplify sheep pox samples. We further confirmed these findings by performing duplex PCR on the same set of samples in which both attachment and fusion gene primers were added together in a single tube reaction. Cycling conditions and annealing temperatures were the same as used for individual A-PCR and F-PCR. The results of duplex PCR were consistent with those of A-PCR and F-PCR carried out independently earlier by us. Duplex

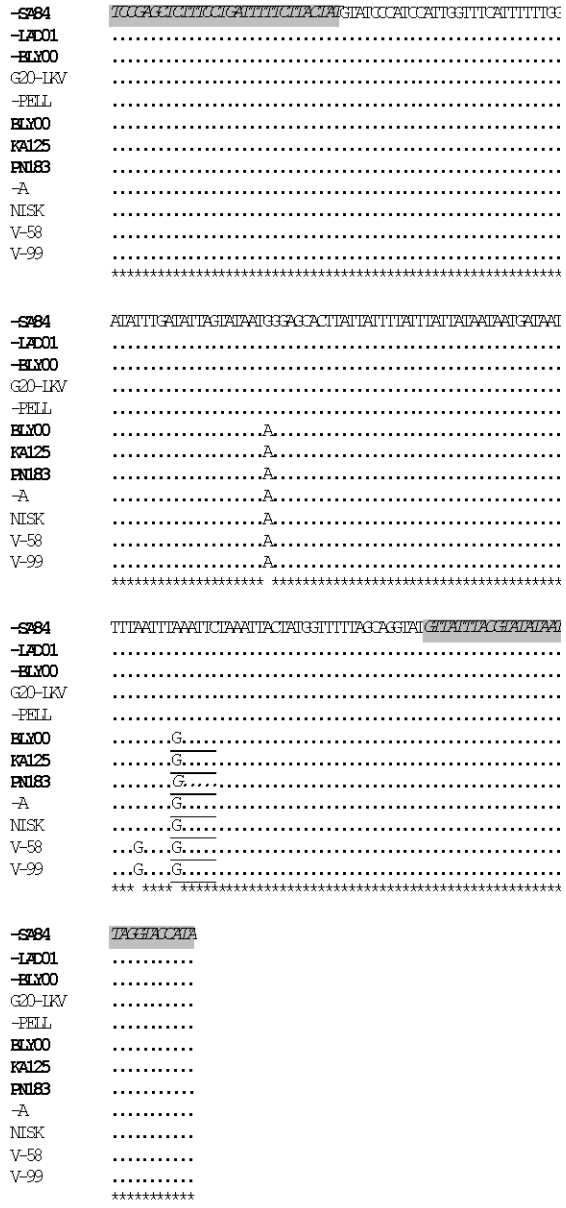

Fig. 3: Multiple sequence alignment of partial attachment gene of capripoxviruses including the Indian isolates using clustalW program 1.81. Sequences of Indian isolates of sheepox and goatpox virus isolates in this study are shown in bold letters"*" indicates highly conserved residue. The nt sequence of the restriction site of *EcoRI* is underlined. Both forward and reverse primer sequences are shown in italics and shaded color

PCR is particularly useful when the SPV samples did not amplify in the F-PCR, as negativity in the test did not necessarily prove so for the capripox. Based on the results, we conclude that duplex PCR can be successfully

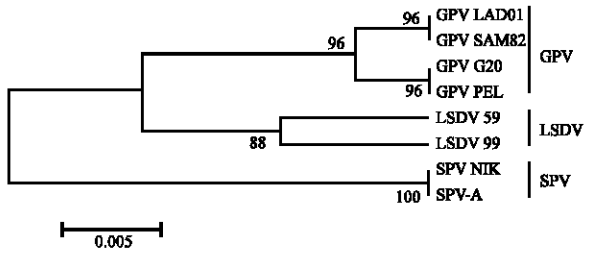

Fig. 4: Phylogenetic analysis of partial fusion gene (411bp excluding the primer sequence) of capipoxviruses. Unrooted tree was constructed by UPGMA method using MEGA 3.0 with 1000 bootstrap values

used for simultaneous detection and differentiation of SP and GP. Results of this PCR were consistent with A-PCR-RFLP method as all the amplicons derived from sheeppox, but not goatpox samples yielded two bands of 129bp and 62 bp on digestion with EcoRI enzyme. Ireland and Binepal[9] have also obtained similar results where in amplicons from all the goat pox samples originating from India, Bangladesh and Iraq were not digestible by EcoRI enzyme. PCR-RFLP of attachment gene amplicons and P32 gene amplicons[14] have been reported for differentiation of capripox virus isolates of sheep and goat origin.

We also cloned and sequenced A-PCR products from three representative GP isolates and three representatives SP isolates. F-PCR products of two isolates of GPV were also cloned and sequenced. Sequencing of A-PCR products showed a length of 191 nucleotides and not 192 bp as reported earlier[9] and digestion of this amplicon yielded two fragments of 129 and 62 bp. Sequences of sheeppox and goatpox virus attachment gene fragments were assembled into multiple alignment sequences to determine the sequence variation among them. It was noticed that a stretch of 129-134 nucleotides GAATTC, which constitutes the recognition sequence of *EcoRI*, was changed to AAATTC in GPV sequence resulting in lack of digestion of all the GPV amplicons (Fig. 3). Fusion gene sequence data obtained from goatpox samples was analyzed by comparison with other capripoxvirus sequences available in the database using NCBI BLAST server[15]. Lack of F gene amplification in sheep pox samples was due to improper annealing of forward primer with GPV template because of mismatch at 3' end of the primer. Substitution of 3' end base of the primer from A to C was consistently present in all the GP virus sequences. Phylogenetic analysis based on partial fusion gene sequence of all the member species of capripoxviruses including our Indian isolates of GPV showed three distinct

clusters of SPV, GPV and LSDV, irrespective of the geographical area of their origin. These observations justify the previous reports[16] that, capripoxviruses although are closely related, phylogenetically they are distinct. Indian isolates of GPV grouped with other GPV strains including GPV Pellore and GPV G20 LKV strains.

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