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Short Communication

Detection of Orf Virus from an Outbreak in Goats and Its Genetic Relation with Other Parapoxviruses

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Abbreviations: BLAST, basic local alignment search tool; BPSV, bovine papular stomatitis virus; CIE, counter-immunoelectrophoresis; dNTP, deoxynucleotide triphosphate; GPY, goat pox virus; HIS, hyperimmune serum; OV, orf virus; PBS, phosphate-buffered saline; PCPV, pseudo cowpox virus; PCR, polymerase chain reaction; PPV, parapoxvirus

INTRODUCTION

Orf, also called contagious ecthyma or contagious pustular dermatitis, is a nonsystemic eruptive skin disease that occurs worldwide in sheep and goats. Orf is caused by orf virus (OV), which is the type species in the *Parapoxvirus* (PPV) genus of *Poxviridae* (Esposito *et al.*, 1995). The genus includes other members of veterinary importance, viz. bovine papular stomatitis virus (BPSV), pseudo cowpox virus (PCPV), PPV of red deer in New Zealand and PPV of grey seal (Robinson and Mercer, 1995; Mercer and Haig, 1999; Becher *et al.*, 2002). The tentative members of the genus cause disease in camels and red squirrels (Dashtseren *et al.*, 1984; Sainsbury and Gurnell, 1995). Parapoxviruses (PPVs) are antigenically and genetically related and have a similar morphology, genomic organization and virulence mechanism (Fleming *et al.*, 1993). Features that distinguish PPVs from other poxvirus genera are the ovoid virion shape, the criss-cross pattern on the particle surface, and the relatively small size and high G + C content of the genome (Moss, 2001; Delhon *et al.*, 2004). The OV is an epitheliotropic virus that generally causes proliferative lesions in the skin of the lips, around the nostrils, oral mucosa and teats of sheep, goats, wild ruminants (e.g. reindeer) and camels (Kummeneje and Krogsrud, 1979; Khalafalla *et al.*, 1994; Haig and Mercer, 1998; de la Concha-Bermejillo, 2003). Both OV and BPSV cause occupational infections in humans, with lesions characterized by large, painful nodules in the hands and, less frequently, the face (Bowman *et al.*, 1981; Meechan and MacLeod, 1992). The precise

geographical distribution of orf is not known, but it is thought to be present in any part of the world where sheep and goats are raised (de la Concha-Bermejillo, 1995).

Orf is recognized in sheep and goats by the appearance of papules, vesicles and rapidly growing scabs in lips and muzzle of the affected animals. In more severe cases, the skin of other areas such as eye, udder or vulva may be affected, and secondary bacterial infections or myasis of the affected area may also occur (Greig *et al.*, 1984; Zamri Saad *et al.*, 1993; Housawi and Abu Elzein, 2000). The disease runs for 3–4 weeks and usually resolves in 2 months. Although it is considered a mild disease, mortality rates up to 10% and 93% have been reported in lambs and kids, respectively (Mazur and Machado, 1989; Gumbrell and McGregor, 1997). In most cases, animals that have been vaccinated or infected with OV are resistant to natural infections. However, outbreaks of orf have also been reported in orf-vaccinated animals (Buddle *et al.*, 1984; Pye, 1990; de la Concha-Bermejillo, 1999), indicating that continuous reinfection with OV is possible. This immune escape may be attributed to evolutionary genetic mechanisms that poxviruses have developed to evade the immune system of the host (Haig *et al.*, 1996, 1997; Deane *et al.*, 2000). The disease is present throughout India, including the Himalayas, but is only occasionally reported (Somvanshi *et al.*, 1987; Batra *et al.*, 1999), probably because of its low severity and marginal economic loss. In this paper, we describe an outbreak of orf in goats and confirm the identity of the virus by amplification of viral DNA and sequencing. This is the first information on genetic relatedness of any Indian orf virus with other isolates around the world.

MATERIALS AND METHODS

The outbreak

The outbreak reported here occurred in January 2001 in a small experimental herd of 34 goats maintained in the Central Himalayas (29°28′42″ N; 79°39′9″ E; altitude 2251 m). These animals were procured from the plains and the outbreak occurred soon after transportation. Animals of all ages, especially the young ones, were affected. Lesions appeared on the junctions of the lips (Figure 1), on the nostrils and, in one animal, on the teats. These lesions turned into scabs at later stage. There was mild increase in the body temperature in young animals. Morbidity was 61% (21 animals were affected out of 34) but there was no mortality. Affected animals were dull and reluctant to take feed during the peak of illness as a result of of lesions on the mouth. Lugol's iodine was applied locally on the lesions. Scabs, formed over the lesions, were collected as a source of virus. The scabs were ground to make a 10% suspension in phosphate-buffered saline (PBS), pH 7.2, clarified by centrifugation and used in immunodiffusion and other tests.

Counter-immunoelectrophoresis

Parapoxvirus antigens were detected in scab materials by counter-immunoelectrophoresis (CIE) using 10% scab suspension against goat poxvirus (GPV) hyperimmune serum (HIS).

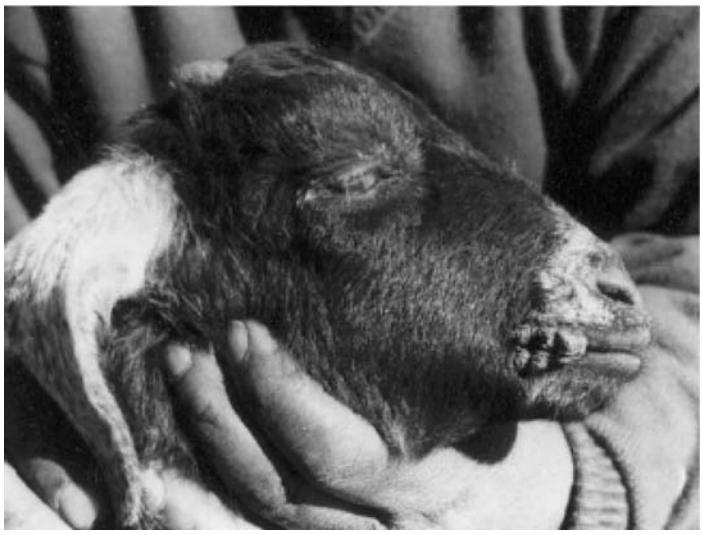

Figure 1. Orf lesions on the oral commissure of an affected goat

Since OV HIS was not available, we used GPV HIS, which cross-reacts with OV antigens. The CIE was performed according to the method described by Sharma and colleagues (1988). Briefly, 1% agarose was prepared in 0.04 mol/L barbital buffer pH 8.6, and 5 ml of molten agarose was poured onto a clean glass microscopic slide. Wells were punched after solidification, and were filled with the respective antigen/antibody and electrophoresed at 20 V/cm for 45 min. A 10% suspension of scab produced by the Sambalpur isolate (Bandyopadhyay *et al.*, 1984) of goat poxvirus (GPV) was used as positive antigen. The HIS was prepared in goat against the same isolate and used as positive antibody. Healthy goat skin was also included as negative antigen.

Polymerase chain reaction (PCR)

The scab materials were also analysed by a diagnostic PCR. DNA was extracted from 100 mg scab material using the QIAamp tissue kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. A set of primers, viz. pan-parapoxvirus primer 1 (PPP-1) and PPP-4 and an inner primer PPP-3 for semi-nested PCR, was used in this study. These primers were designed by Inoshima and colleagues (2000) based on the previously published sequence of the *B2L* gene of OV strain NZ2, which is a homologue of vaccinia virus major envelope antigen p37K (Sullivan *et al.*, 1994). The sequences of PPP-1, PPP-4 and PPP-3 were 5′-gtc gtc cac gat gag cag ct-3′; 5′-tac gtg gga agc gcc tcg ct-3′ and 5′-gcg agt ccg aga aga ata cg-3′, respectively.

PCR was carried out following the method described earlier (Inoshima *et al.*, 2000). Briefly, DNA from 100 mg scab material was added to 50 µl reaction mixture containing 0.2 $\mu mol/L$ primers, 200 $\mu mol/L$ each dNTP, 10 mmol/L Tris-HCI (pH 8.3), 50 mmol/L

KC1, 1.5 mmol/L $MgCl_2$, 2 units of *Taq* DNA polymerase (Promega, Madison, WI, USA). DNA was amplified in a thermal cycler (DNA Engine, MJ Research, MA, USA) by a two-step reaction: 95°C for 9 min, and five cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min, and then 25 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. Semi-nested PCR was carried out using 5 µl of the first PCR product in the same conditions with PPP-3 and PPP-4 primers. After amplification, a 5 µl aliquot was electrophoresed through 1% agarose gel and stained with ethidium bromide for visualization of the expected amplicon.

Cloning and sequencing of the PCR amplicon and analyses of sequence data

The amplicons generated by semi-nested PCR (using PPP-3 and PPP-4 primers) were cloned into pGEM-T Easy vector (Promega) following the manufacturer's instructions. The presence of the insert (235 bp) was determined by restriction endonuclease (RE, *Eco*RI) analyses of recombinant plasmids. Nucleotide sequencing of three positive clones, generated from three different PCRs, was done in both orientations by automated sequencer (ABI Prism, 3100, IL, USA).

Sequences were analysed by comparison with sequences of different parapoxviruses available in databases using the online BLAST server (Altschul et al., 1997). The multiple sequence alignment was carried out by CLUSTAL W algorithm (Thompson *et al.*, 1994) and edited manually. Phylogenetic analysis was conducted using MEGA version 2.1 (Kumar *et al.*, 2001). The Tamura Nei (1993) model of nucleotide substitution with gamma-distribution of among-site rate heterogeneity (with eight categories) (termed the TrN+G model) available in MEGA was used to construct the trees. The tree topologies were evaluated using 10000 replicates of the dataset.

RESULTS AND DISCUSSION

In the CIE test, cross-reaction was observed between goat pox virus hyperimmune serum (GPV HIS) and clinical samples (scabs) in the form of precipitin bands, which indicate the presence of antigens in the scab materials (data not shown). There were precipitin bands in positive control and no band in negative control as expected. Orf virus (OV) shares a precipitating polypeptide of the same molecular weight as goat pox virus (Kitching *et al.*, 1986). The cross-reactions among the OV, goat pox virus (GPV) and sheep pox virus (SPV) antigens have previously been studied and it has been demonstrated that GPV antiserum develops precipitin lines with OV antigen in immunodiffusion tests (Sharma and Dhanda, 1971; Subbarao *et al.*, 1984). Since antiserum against OV was not available to us, GPV antisera was used, where precipitin bands in CIE gave indication of the presence of antigen (either orf or goat pox) scabs. Although immunodiffusion test cannot be used for diagnosis of orf because of the cross-reaction, it could nevertheless be of considerable use in epidemiological surveys when large numbers of samples are to be examined. The presence of antigen in scabs examined in this study is suggestive of parapoxvirus infection, which was further confirmed as orf by PCR and nucleotide sequencing.

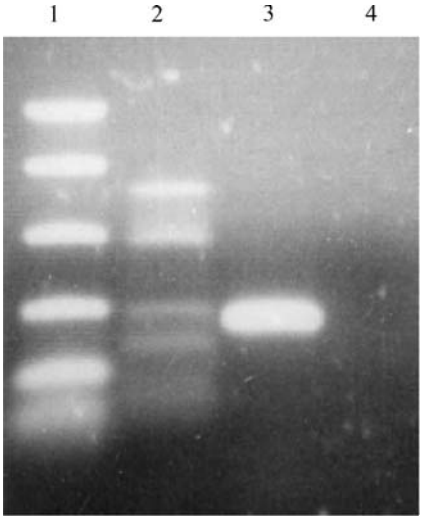

Figure 2. Detection of orf virus DNA in scab materials by PCR. Lane 1, molecular weight markers 1000, 750, 500, 300, 150 and 50 bp; lane 2, first PCR with PPP-1 and PPP-4 primers, showing non-specific amplifications; lane 3, appropriately sized (235 bp) product in seminested PCR with PPP-3 and PPP-4 primers; lane 4, DNA isolated from goat pox scab with semi-nested primers, showing no amplification

We have analysed scabs for the presence of orf virus DNA by PCR. An appropriately sized product (235 bp) was amplified from the scab materials by semi-nested PCR (Figure 2, lane 3); however, there was no specific amplification in the first PCR using PPP-1 and PPP-4 primers despite repeated attempts.

The same primer pair had been used successfully to amplify a specific fragment (594 bp) from clinical samples for the diagnosis of parapoxvirus infection in reindeer (Tryland *et al.*, 2001). It may not be always possible to obtain specific amplification from clinical samples using this primer pair, but use of semi-nested primers ensured the detection of parapoxvirus in our samples. Semi-nested PCR could detect low copy number of viral DNA and was found to be more efficient for diagnosis of parapoxvirus in clinical samples (Inoshima *et al.*, 2000). Semi-nested PCR was also carried out using DNA isolated from goat pox-positive scab as a negative control and, as expected, there was no amplification. This also indicates that the pan-parapox primers developed by Inoshima and colleagues (2000) are specific to parapoxvirus and do not anneal to goatpox virus DNA.

The amplicon generated by semi-nested PCR was cloned, sequenced and analysed to confirm the identity of the virus. The partial sequence (235 bp) of the major envelope gene of this virus (designated as OV-MU) was submitted to GenBank (accession no. AY 545034) and compared with 12 different parapoxvirus strains, OV-V (AY278209), OV-IA82 (AY386263), OV-MO (AY424969), OV-sheep (AY424970), OV-takin (AY424971), OV-NZ-2 (U06671), OV-SAOO (AY278208), F92.849R (AY453659), PCPV-TQ (AY424972), F00.120R (AY453656), BPSV-ARO2 (AY386265) and RDDPV (AY453655), for phylo-

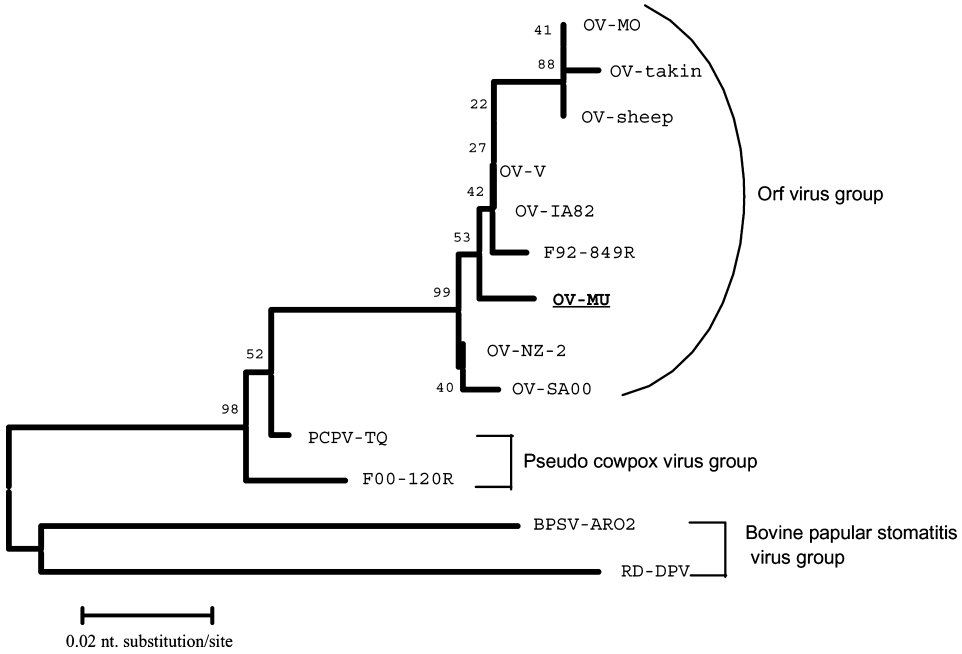

Figure 3. Phylogenetic clustering of different parapoxviruses based on the partial nucleotide sequence of the major envelop gene. The scale bar beneath the tree indicates nucleotide substitutions per site. OV-MU in the tree shared branching with other published orf viruses. Note that OV-V is a vaccine strain used in Texas (Hussain and Burger, 1989; Guo *et al.*, 2003). The OV-MO, OV-sheep, OV-takin and PCPV-TQ were isolated from musk ox, sheep, takin and tillquist, respectively (Guo *et al.*, 2004). The strain OV-SAOO was isolated in Texas from a kid with severe multifocal, proliferative dermatitis and propagated in Madin–Darby ovine kidney cells (Guo *et al.*, 2003). The strains OV-IA82 and PBSV-AR02 were isolated from lamb and calf, respectively in the United States (Delhon *et al.*, 2004). The OV-NZ-2 was isolated from sheep in New Zealand (Robinson *et al.*, 1982). The RD-DPV is a parapoxvirus (DPV strain) of red deer in New Zealand (Robinson and Mercer, 1995; Tikkanen *et al.*, 2004). The strains F92.849R and F00.120R are parapoxviruses of reindeer. The former is an orf virus and the latter is closely related to PCPV (Tikkanen *et al.*, 2004)

genetic analysis. The characteristics of the major envelope gene of these strains have been described previously (Sullivan *et al.*, 1994; Guo *et al.*, 2003, 2004; Delhon *et al*, 2004; Tikkanen *et al.*, 2004). The phylogenetic tree based on the 235 bp nucleotide sequence of the envelope gene of various parapoxviruses showed three distinct groupings corresponding to three virus species of the genus, which was supported by high bootstrap confidence (Figure 3).

The grouping pattern in which all the parapoxviruses – orf virus, PCPV and BPSV – formed separate clusters was consistent with recent report of Tikkanen and colleagues (2004). In the phylogenetic tree, the virus under study (OV-MU) clustered only with orf viruses. This was further supported at the nucleotide level, where the present virus was

1.3–3.0%, 3.9–5.3% and 15.3–16.2% divergent in relation to orf, PCPV and BPSV group of viruses respectively. This also shows that OV-MU virus is more closely related to the orf group of virus than to other species of parapoxviruses. However, these data need to be further supported by sequence analysis of full-length envelope gene of OV-MU and other isolates from this geographical region. The virus was 1.3% divergent in nucleotide sequence from the orf vaccine strain (OV-V) and was different from other orf viruses from different countries compared in the analysis.

In the present report, we describe a mild outbreak of stomatitis in goats and identify the causative agent as an orf virus that is genetically closely related to other orf viruses from different geographical regions. The phylogenetic analyses indicate that the virus is an orf virus distinct from other orf virus strains. Although orf is endemic in India, before this report there was no information on genetic relatedness of any Indian OV to other isolates around the world.

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