Isolation and molecular characterization of contagious pustular dermatitis virus from Rajasthan, India

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Abstract This article describes the isolation and identification of contagious pustular dermatitis virus/orf virus (ORFV) from an outbreak of contagious pustular dermatitis (orf) in flocks of goats, in the north western region of India (Rajasthan). The virus was isolated in Vero cell cultures from scab and swab suspensions and has been identified using GIF/IL-2 and B2L gene specific primers in PCR and sequencing. The virus showed high nucleotide identity with previously reported Chinese, far eastern, Brazilian and Indian isolates. This report described the use of molecular tools for fast, reliable and confirmatory diagnosis of ORFV infection.

Keywords *Parapoxvirus* · *Poxviridae* · Orf · Contagious pustular dermatitis · CPD · OPD · ORF · Ovine pustular dermatitis · Epidemiology · Rajasthan · India

Orf or contagious ecthyma (CE) or contagious pustular dermatitis (CPD) or ovine pustular dermatitis (OPD) is a specific dermal infection of sheep and goats [7], which is caused by a *Parapoxvirus* [the contagious pustular dermatitis virus (CPDV) or orf virus (ORFV)], of the subfamily *Chordopoxvirinae*, family *Poxviridae* [13]. The genus *Parapoxvirus* also includes pseudocowpox virus (PCPV) and bovine papular stomatitis virus (BPSV) in cattle, squirrel *parapoxvirus* (SPPV), and *parapoxvirus* of red deer in New Zealand (PVNZ). It is a nonsystematic

eruptive skin disease having public health importance. The classical clinical disease is usually manifested as scabby lesions around the mouth [6, 17]. The lesions are initially erythematous spots or swelling, and this is followed by formation of papules which become bigger and pustular with a yellowish creamy appearance. These papules progress to develop into scabs [10]. Other forms of the disease are also observed. These may involve other parts of the skin, including the face, udders, ears and under the tail [9]. Generalized infection has also been reported [1]. Although CE infection in sheep and goats is usually self-limiting, sometimes it can be a long-lasting condition making affected animals susceptible to other bacterial conditions [1]. Severe and mild orf outbreaks have been reported from different parts of the world and annual losses as a result of natural orf infection is estimated to be millions of dollars [16].

The genome of ORFV is a linear double-stranded (ds) DNA molecule of ~135 kb in length. The central part of the genome contains the highly conserved genes which are essential for viral replication and virus morphogenesis, and variable genes are located at the terminal ends are mainly responsible for virus virulence and pathogenesis [5]. The envelope gene (B2L) of ORFV encodes a highly immunogenic envelope protein of about 42 kDa. The B2L, DNA polymerase, GIF/IL-2 and VIR genes have been used in studies related to genetic variation and molecular epidemiology [3, 12, 14, 15].

Here the authors report an outbreak of CE involving goats in the Chokri village of Sikar district of Rajasthan, in which some animals suffered from severe complications of the condition. The causative agent (CPDV/ORFV) was isolated in cell culture, identified and confirmed in the outbreak samples using molecular tools. This study on the genetic diversity of CPDV in India may contribute to an improved

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understanding of CPDV pathogenesis, infection biology and epidemiology, and facilitate CPDV vaccine development.

In the early summer of 2013 (April–June), several outbreaks of CE affected goats in Rajasthan, the north western region of India. Although the disease in most cases resembled the classical form (scabby mouth), as described earlier [9], some goats (10 %) were affected by an aggravated form. Clinical examination of these animals revealed elevated temperatures which reached up to 41 °C. The lesions were severe, but were confined to the skin around the mouth, the gums and the inner surface of the lips. The affected goats were aged between three and twelve months. Nearby villages in the radius of 30 km (e.g. Chala, Khandela, Govindpura and Bhagega) were also affected.

The disease was observed in five flocks of local breeds of goats (Jhakrana and Beetal) in Chokri village of Sikar district of Rajasthan State. Flock A had 100 goats, out of which 60 animals were affected and 6 died also. Three scab samples were taken from this flock. A scab sample (designated as CPDV/IND2013/02) was taken from lower jaw and lips from a 3 years old goat showing high temperature (41 °C). Another scab sample (designated as CPDV/IND2013/03) was taken from lower lip of a ~2 years old animal with acute diarrhea. The third scab sample (designated as CPDV/IND2013/04) was taken from knee joint of an animal of 3 years of age of the same flock showing signs of pneumonia but no diarrhea. Additionally, four nasal swab samples were taken from flocks B (from a 3 years old goat—CPDV/IND2013/05), C (from a 5 years old goat—CPDV/IND2013/06), D (from a 13 years old goat—CPDV/IND2013/07), and E (from a 9 years old goat—CPDV/IND2013/08). All of these animals were showing characteristic signs of CPD, papules on mouth with pneumonia and/or diarrhea. These animals were not previously immunized against CE.

The sick animals were given intramuscular supportive treatment with 1 % dextrose and multi-vitamins by local veterinarians. Betadine dressing was applied to the wounds to prevent secondary infections. Additionally enrofloxacin was also given at the dose rate of 2.5 mg/kg B. Wt., per day for 5 days to avoid bacterial infection. The animals also received Melonex (NSAID) at the dose rate of 0.2–0.5 mg/kg B. Wt., in order to reduce pain.

All seven samples consisting of three scabs and four nasal swabs were triturated and homogenized (scabs) or suspended (swabs) in PBS pH 7.4 with antibiotics (penicillin 1,000 IU/ml, streptomycin 1,000 µg/ml, amphotericin B 25 mg/ml and kanamycin 500 µg/ml) from Sigma Chemicals making up a 10 % (w/v) solution. The 10 % suspension was clarified by centrifugation at 2,500×g for 15 min. The supernatant was collected and stored at –80 °C until further use. Part of this suspension was used for DNA isolation for PCR and part of this was inoculated

onto to Vero cell culture after filtration through 0.22 micron filter for virus isolation [9]. Two scab samples CPDV/IND2013/03 and CPDV/IND2013/04 were pooled together and also four swab samples (CPDV/IND2013/05, CPDV/IND2013/06 CPDV/IND2013/07, and CPDV/IND2013/08) were pooled for passage in cell culture.

Monolayers of Vero cells grown in 25 cm² tissue culture flasks were inoculated with sample supernatant using two flasks per sample. Following incubation at 37 °C for 1 h maintenance media [Eagle MEM (M0769—Sigma)] without fetal calf serum (FCS), but having penicillin (10,000 UI/ml) and streptomycin (100 µg/ml), was added. The flasks were incubated at 37 °C and were observed daily for the appearance of cytopathic effects (CPE) for up to 7 days post-inoculation.

The viral DNA was extracted from clinical material (scabs and swabs) and cell culture adapted virus isolates using phenol/chloroform method as described by Sambrook et al. [18], and stored at –20 °C until further use. The major envelop membrane glycoprotein (B2L) gene of the virus was used in the molecular diagnosis, characterization and phylogenetic analysis studies. For PCR the standard protocol as described by Inoshima et al. [11] was followed. This protocol amplifies partial sequences of the B2L gene and uses a set of 3 primers in a semi-nested PCR format. For first round of amplification a set of pan-*parapoxvirus* primer 1 (PPP-1) and PPP-4, were used to generate an amplicon of 594 bp. For semi-nested PCR, an inner primer PPP-3 was used in combination with PPP-4 to produce an expected product size of 235 bp (Table 1).

Two more sets of primers were designed for full-length (1,137 bp) or near full length amplification of B2L gene (912 bp) as listed in Table 1. For this PCR, following cycling parameters: 95 °C for 3 min, 35 cycles of denaturation (94 °C, 1 min), annealing (54 °C, 1 min) and extension (72 °C, 1 min) were used. ORFV GIF/IL-2 gene (the virulence factor found only in *Parapoxviruses*) specific primers as reported by Ramesh et al. [15] were also used for its confirmatory diagnosis (Table 1).

PCR amplified products were gel purified using Qiaquick gel extraction kit (Qiagen) and sequenced in Automated genetic analyzer (ABI 3130XL) using BigDye terminator v3.1 cycle sequencing kit from Applied Biosystems. Cycle sequencing PCR was performed using standard protocol. The sequence data generated was assembled using SeqMan programme of the Lasergene software ver 5.0 and was subjected to BLAST analysis for comparison with other available B2L gene sequences in Genbank. Sequences were aligned and phylogenetic analysis was performed using MEGA 5.0 programme [19]. The nucleotide sequence identities were calculated using MEGA 5.0 programme.

The CE outbreaks described here occurred in goat flocks of whole village with high morbidity (40 %) and

Table 1 Primers for the amplification of ORFV specific genes

Primer-pair designation	Primer name	Primer sequence (5'-3')	Product size (bp)
Pair 1	ORFV/B2L/1-23F	ATGTSGSCGTTCTHCTYCATCCC	1,137
	ORFV/B2L/1137-1115R	TTWWTTATTGGYTTGCARRABT	
Pair 2	ORFV/B2L/142-161F	TTGTACATCTGCAGCTTCTG	912
	ORFV/B2L/1054-1036R	CGTTCACGCTCACRAAGGC	
Pair 3 [15]	ORFV/IL-2/F	GCTCTAGGAAAGATGGCGTG	408
	ORFV/IL-2/R	GTACTCCTGGCTGAAGAGCG	
Pair 4 [11]	PPP-1/B2L	GTCGTCCACGATGAGCAGCT	594
	PPP-4/B2L	TACGTGGGAAGCGCCTCGCT	
Pair 5 [11]	PPP-3/B2L	GCGAGTCCGAGAAGAATACG	235, when used in combination with PPP-4

comparatively low (10 %) mortality. There was development of classical clinical signs including lesions on the lips and muzzle with diarrhea, pyrexia, inappetence and pneumonia, which are strong evidence of an ORFV infection (data not shown). Diagnosis of CPD is generally done on the basis of presence of characteristic skin lesions, electron microscopy studies and serology [21]. However, molecular tools involving PCR and sequencing are more rapid, sensitive and give confirmatory diagnosis of the presence of CPDV DNA in the samples. B2L gene is highly conserved and immunogenic enveloped protein of the virus and is a very good target for development of diagnostic assays for identification of these viruses.

All seven samples consisting of three scabs and four nasal swabs were processed for adaptation to Vero cells. Inoculation of scabs and swab supernatants on Vero cell cultures revealed a cytopathic effect (CPE) in the form of cell rounding and enlargement, pyknosis, granularity of the cytoplasm and cell detachment. In each sample, virus induced CPE were noted in the Vero cells from the first passage onwards. These changes in cells started about the third or fourth day of incubation and usually resulted in almost complete destruction of the cell sheet by the seventh day. When 100 % CPE was observed, the flasks were frozen at -20°C (after pH adjustment) (data not shown). Control cells remained healthy. The virus was harvested after two freeze–thaw cycles. Five blind passages were carried out for all samples.

The total viral DNA extracted from infected Vero cells (from all five passages of each sample) harvested at 100 % CPE, as well as directly from scab and swab samples when tested using semi-nested B2L gene specific PCR as well as full length B2L gene amplification PCR, produced correct sized B2L gene amplicon of 235 and 1,137 bp respectively (Fig. 1). The presence of ORFV in clinical samples was also confirmed using GIF/IL-2 gene specific PCR yielding correct sized 408 bp amplicon (Fig. 1). The PCR results confirmed that virus present in these samples has adapted

well to Vero cells. The 1,137 bp PCR amplicon from CPDV/IND2013/02 was sequenced and full length B2L gene contig sequence obtained was submitted to Genbank (Accession number KF441714).

Nucleotide sequence and the phylogenetic analysis of B2L gene of CPDV/IND2013/02 revealed high conservation in B2L gene. Neighbour joining tree grouped all global CPDV isolates ($n = 71$) into one major cluster/group having several small sub-clades with overall >96 % nucleotide (nt) identity amongst them (Fig. 2). ORFV strains (including CPDV/IND2013/02) showed 84–86 % nt identities with BPSV, 93–96 % with PCPV, 43–46 % with PVNZ. CPDV/IND2013/02 showed lowest nucleotide identities (96 %) with four Chinese strains (Accession nos: JQ904795, JQ904799, HQ694772 and JQ619903) while highest nucleotide identities (99 %) with some other Chinese, far eastern, Brazilian and Indian isolates (Accession nos: JQ904789, GU320351, GQ328006, EU327506, JN613810 and DQ263304). These analyses also showed that the Indian CPDV strains including CPDV/IND2013/02 are distinct from other members of the clade and fall separately into different sub-groups within a single clade. CPDV/IND2013/02 showed 98 % nt identity with a most recent isolate from Central Institute for Research on Goats (CIRG), Makhdoom, Mathura, (Accession no. KC992325) [12]. Although CPDV strains isolated from different hosts grouped very closely in the phylogenetic tree showing very high identity levels, Kumar et al. [12] have identified a critical amino acid (aa) residue at position 249 in the B2L gene. All the sheep strains had serine (249S) whereas all the goat strains except few (JN088053, JQ349520, AY278209, JN846834, JQ040300, and DQ263303) had glycine (249G) at this position. The deduced aa sequence from B2L gene of CPDV/IND2013/02 also had a serine at this site. The B2L gene based phylogenetic analyses presented here revealed that four species of *parapoxviruses* (ORFV, PCPV, BPSV, PVNZ) form separate clusters with nucleotide identity levels in the range of 46–95 %.

Fig. 1 **a** PCR amplification of B2L and GIF/IL-2 genes of CPDV. PCR amplification of B2L gene using PPP1 and PPP4 primer-pair (Pair 4) listed by Inoshima et al. [11] showing amplification of 594 bp products (Table 1). **b** PCR amplification of B2L gene using PPP3 and PPP4 primer-pair (Pair 5) listed by Inoshima et al. [11] showing amplification of 235 bp products (Table 1). **c** PCR amplification of B2L gene using newly designed primer pair 1 showing amplification

of full length 1,137 bp products (Table 1). **d** PCR amplification of GIF/IL-2 gene using primer pair (Pair 3) listed by Ramesh et al. [15] showing amplification of 408 bp products (Table 1). Lane 1 CPDV/IND2013/02 (Scab), Lane 2 CPDV/IND2013/05 (Swab), Lane 3 CPDV/IND2013/03+CPDV/IND2013/04 (Pooled scabs at P5). M1 100 bp DNA ladder (MBI Fermentas); M2 1 Kb DNA ladder (Invitrogen); +C autogenous vaccine strain; -C negative control

Fig. 2 Unrooted neighbour-joining phylogenetic tree using CPDV strain sequences [full length sequences (1,137 bp) of B2L gene]. The tree was constructed using a p-distance algorithm and pairwise deletion parameters. Texas show GenBank accession numbers and geographic origin of strains that were compared. Bootstrap values (%) are represented at each tree node. Node support was assessed with 500 bootstrap pseudo-replicates. The CPDV/IND2013/02 strain characterized in this study is indicated in blue box. (Color figure online)

Recently there have been increased reports of CPDV infection worldwide and is enzootic in India [3, 12, 20]. More recently an outbreak of CPDV infection was reported

in Muzzaffarnagari sheep flock of the CIRG, Makhdoom, Mathura, with about 10 % morbidity and no mortality [12]. ORFV has been extensively investigated over recent years,

due to its zoonotic importance and ability to cross-infect other species by sporadically evasive mechanisms, which the virus has developed to adapt and grow in the presence of an active immune response. This helps to explain the ability of the virus to repeatedly reinfect the same host, a phenomenon also observed in an outbreak of ORFV infection during December 2012, followed by in August 2013 at the Central Sheep Breeding Farm (CSBF), Hisar, Haryana (Aman Kumar, personal communication). Exposure of Animals to stress or immune-suppression as a result of therapy or primary viral infection can accentuate the severity of disease [8]. Because of the persistence or repetition of ORFV infections, it is very difficult to eliminate this virus and hence CPDV infection is considered a re-emerging disease in India and other countries [2, 4].

Given the increased incidences and economic importance of CPDV in small ruminant population in India, it will be worthwhile to obtain more epidemiological information about this disease for effective surveillance, to carefully monitor and handle the outbreaks and to develop an appropriate vaccine. This report adds additional data on the molecular epidemiology of CPDV and described the use of molecular tools for fast, reliable and confirmatory diagnosis of CPDV infection in goat population of Sikar district of Rajasthan.

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