SHORT COMMUNICATION

Detection and phylogenetic analysis of Orf virus in Kashmir Himalayas

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Abstract Orf virus (ORFV) is a zoonotic pathogen that primarily infects sheep and goats, and is responsible for significant economic losses. ORFV is endemic in all the major sheep and goat rearing areas of the world including Indian subcontinent. However, the nature of ORFV circulating among sheep and goat in Kashmir Himalayas has not yet been characterized. In the present study, we describe natural outbreaks of ORFV in sheep and goats of Kashmir Himalayas. We detected the presence of ORFV in the scab lesion by PCR amplification of the major envelope protein (B2L) gene. We sequenced the virus interferon resistance (VIR) gene and determined their phylogenetic relationship with that of the published reference sequences. Phylogenetic analysis based on VIR gene revealed that the ORFV isolates from Kashmir Himalayas separated into main two clusters. The sheep isolates showed genetic homology with

the sheep strains reported from Greece and Italy, whereas the goat-specific strain show homology with the goat strains reported from China. This study demonstrates the presence of ORFV infection in sheep and goats, and report first phylogenetic analysis of the ORFV strains prevalent in the Kashmir Himalayas.

Keywords Orf virus · Contagious ecthyma · Sheep · Goat · Virus Interferon Resistance (VIR) gene · Phylogenetic analysis

Orf virus (ORFV) is a prototype member of the genus *Parapoxvirus* in the subfamily *Chordopoxvirinae* and family *Poxviridae*. It is a causative agent of a highly contagious exanthemic disease, known as Orf or Contagious Ecthyma, in small ruminants especially sheep and goats, and can sporadically infect humans. ORFV infection in the sheep and goat causes proliferative and often self-limiting scab lesions in the skin of the lips, the muzzle and around the nostrils, which may occasionally extend to oral mucosa, feet and teats. The clinical disease usually resolve spontaneously within 1–2 months, but can lead to a significant mortality in lambs and kids that are unable to feed themselves due to oral lesions or associated secondary infections [17]. ORFV is distributed worldwide and several outbreaks have been reported from different part of the India [2, 6, 11]. ORFV has a significant economic impact through hampering optimum productivity of the affected animals, reduced market value of the animal products, and disruption of global trade in animals and animal products.

Like other parapoxviruses, ORFV is morphologically distinguished from that of poxviruses in its relatively small size, crisscross pattern on the particle surface, and high GC content of the genome. ORFV genome consists of a linear

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double stranded DNA (~ 135 kbp in size) having 288 putative genes on both the strands with a bi-directional orientation. The central part of the genome harbors highly conserved genes involved in the viral replication and morphogenesis, and the terminal ends contain variable genes that are responsible for the virulence and pathogenesis [16]. ORFV is typically detected in clinical samples by a conventional polymerase chain reaction (PCR) assays based on the amplification of B2L gene [18]. The B2L gene encodes a conserved and highly immunogenic envelope protein of about 42 kDa size. Virus interferon resistance (VIR) gene is located in the left terminus of the genome, encoding a dsRNA-binding protein that inhibits the antiviral activity of interferons during infection. VIR gene has been used to study the genetic variation and the molecular epidemiology of the ORFV strains [8, 9, 12, 15].

Kashmir is a beautiful valley in the Indian Himalayan region located between 33°20'–34°54' north latitude and 73°55'–75°35' east longitude, bound on the southwest by the Pir Panjal Range and on the northeast by the main Himalayan range. The valley is mostly mountainous, covering an area of 15,948 km², and has abundant green alpine pastures and meadow lands. Sheep and goat rearing is the main economic activity and source of livelihood for many ethnic tribes including Gujjars and Bhakarwals. ORFV is endemic in small ruminants of Kashmir and currently no vaccination program is performed to control the disease. Despite Orf disease being reported in the sheep and goat of Kashmir [3, 5], the nature of circulating ORFV strains remains unknown. Understanding the molecular characteristic of the circulating ORFV strains is crucial for their control and even eradication. Here, we detected ORFV in the sheep and goat of Kashmir, and determined their genetic diversity by comparing the full-length VIR gene. This is the first report of phylogenetic analysis of the ORFV strains prevalent in the Kashmir Himalayas.

The natural outbreaks of ORFV described here were recorded at four isolated sheep farms in Kashmir during 2014–2015. The study also included samples from Orf cases of sheep and goat presented for treatment at the Teaching Veterinary Clinical Complex (TVCC) of the FVSc & AH during May and June 2014 (Table 1). Of the 662 total sheep at four farms, 108 developed scab lesions suggestive of ORFV infection (Fig. 1a, b). Majority of the affected the sheep had lesions on the oral commissures, lips, muzzle and nose. Few cases developed severe diffuse proliferative lesions on muzzle that extended into the oral mucosa. No observable lesions were recorded in other parts of the body. Most of the affected sheep were below 6 month of age. Although high mortality rate among young non-immune animals has been reported, we recorded 5 of the 108 affected lambs, aged 1–2 months, had died due to inanition caused by severe oral lesions. This accounted for

disease morbidity rate of 26.66% (108/662) and the case fatality rate of 4.62% (5/108). We also did not observe any zoonotic ORFV infection among the farm workers during this study. Even though zoonosis has been reported in literature, the mere absence of disease or lesions in the workers does not completely rule out the infection. Human infections may be self-limiting and may occur without the presence of severe disease [1].

The clinical diagnosis of the ORFV infection is possible but it can be erroneous for differentiating from other exanthemic disease including Ulcerative Dermatitis, Blue Tongue and Foot-and-Mouth Disease. The clinical diagnosis may be complicated when the lesions become secondarily infected. Demonstration of the ORFV in the scab material by electron microscopy or in the cell culture are considered gold standards for diagnosis, but they are time-consuming, expensive and non-viable in certain settings [4]. Currently, PCR based methods have emerged more reliable and robust tool for diagnosis of the ORFV infection in animals (reviewed in [7, 10]). In the present study, we used PCR assay using primer pair targeting full-length major envelope protein (B2L) gene [6]. Briefly, the dried scab lesions were collected from clinically affected sheep or goat and stored in a – 20 °C freezer until processed. The scab samples (~ 25 mg) were mechanically homogenized in a 250 µl of phosphate buffered saline (PBS) in a tube using Polytron[®] tissue homogenizer (Kinematica, USA). The homogenates were digested by proteinase K at 55 °C for 3 h and processed for DNA extraction using Wizard[®] Genomic DNA Purification Kit (Promega, USA) as instructed by the manufacturer. The extracted DNA was suspended in 50 µl of nuclease free water and stored in – 70 °C until use. For B2L gene amplification, PCR was performed in a thermocycler using 2 µl of DNA as template in a 25 µl reaction volume containing 10 pmol of each primer and 1 U Taq DNA polymerase (Fermentas, USA). The thermal profile used for the PCR was to heat samples to 94 °C for 3 min, followed by 30 cycles of 1 min at 94 °C, 1 min at 52 °C and 1 min at 72 °C, with the final extension at 72 °C for 7 min. Of total 36 scab samples screened, 31 (86.11%) came positive for B2L gene amplification (Fig. 1c). Earlier, Hosamani et al. [6] reported 100% detection rate of the ORFV in clinical samples in the PCR assay using the same primer sets. The low detection rate in our case may be attributed to the presence of PCR inhibitors in scab material, sequence divergence in the B2L gene or the larger size of the PCR amplicon. A sequence divergence of 15.6% has been reported in B2L gene across the members of Parapoxvirus [14] which may compromise the detection rate of the PCR.

To amplify the full length VIR gene (555 bp), a 5 µl DNA samples were subjected to PCR in a 50 µl volume using primer pair described earlier [13]. The thermal profile

Table 1 Details of the ORFV outbreak investigated in the present study

Location	Species affected	Collection date	Flock size	No. of clinical cases	No. of deaths	No. of samples collected	No. of samples detected positive in PCR	Designation of isolate	Accession no.
1. Sheep Breeding Farm, Dachigam	Sheep	April 4, 2015	135	36	0	9	9	SBF/Dachigam-01/Sheep	KU672677
2. Sheep Breeding Farm, Goabal	Sheep	April 1, 2015	187	28	2	10	7	SBF/Goabal-01/Sheep	KU672680
								SBF/Goabal-02/Sheep	KU672681
3. Bilal's Sheep Farm, Nishat	Sheep	April 2, 2015	151	25	0	8	6	BSF/Nishat-01/Sheep	KU672684
								BSF/Nishat-01/Sheep	KU672685
4. Mountain Research Center for Sheep and Goat, Shuhama	Sheep	March 14, 2014	189	19	3	4	4	MRSG/Shuhama-01/Sheep	KU672686
								MRSG/Shuhama-02/Sheep	KU672687
5. Teaching Veterinary Clinical Complex, Shuhama ^a	Sheep	May 10, 2014	–	1	0	1	1	TVCC/Shuhama-01/Sheep	KU672678
	Sheep	June 10, 2014	–	2	0	2	2	TVCC/Shuhama-02/Sheep	KU672679
	Goat	June 3, 2014	–	2	0	2	2	TVCC/Shuhama-01/Goat	KU672682
								TVCC/Shuhama-02/Goat	KU672683

^aThese represent ORFV case presented for treatment to outdoor clinics at TVCC, FVSc & AH, Shuhama

Fig. 1 Representative clinical cases of ORFV infection. **a** Sheep with scab lesions on lips and oral commissures (SBF/Dachigam-01/Sheep). **b** Sheep with scab lesion that extend up to nostrils (SBF/Dachigam-02/Sheep). **c** PCR detection of ORFV in scab material by PCR amplification of B2L gene using specific primers. The specific PCR

product was visualized in ethidium bromide stained agarose (1%) gel. Lane 1: No specific PCR amplification in the sample, lane 2–5: PCR product of ~ 1200 bp from ORFV positive scab samples, lane 6: Negative control PCR reaction that contained no template DNA, and lane M: 100 bp molecular weight markers (Fermentas, USA)

for VIR amplification was to heat samples to 95 °C for 5 min, followed by 30 cycles of 30 s at 95 °C, 40 s at 52 °C and 30 s at 72 °C, with the final extension at 72 °C for 10 min. The full-length VIR gene product were purified and cloned into the pGEMTeasy plasmid vector (Promega, USA) as per the manufacturer's instructions. The plasmid clones were sequenced from both end using M13 universal primers in an automated DNA sequencer (ABI PRISM 3100, Perkin-Elmer) at CCMB, Hyderabad. The 11 VIR gene sequences were submitted to GenBank with accession number from KU672677 to KU672687.

To analyze the diversity of ORFV prevalent in the Kashmir Himalayas, complete VIR gene sequences of ORFV strains (n = 72) reported from across the world were obtained from GenBank (Supplementary Table 1). The sequences were analyzed and aligned using the ClustalW multiple alignment algorithms in the MEGA7 software suite [10]. VIR sequence alignment revealed that the maximum nucleotide and amino acid variation in the gene was 6.1 and 10.9%, respectively. Among the eleven isolates of this study, the nucleotide and amino acid sequences homology was at least 94.7 and 90.7%, respectively. There was no deletion or insertional mutation within the open read frame, except that a 61 bp nucleotide fragment was additional to the 3' sequence of VIR gene in SBF/Goabal-02 strain. All the nucleotide changes were substitutions, many of which led to the amino acid change in the translated proteins i.e., the mutations were non synonymous in nature.

Phylogenetic analysis of the VIR gene sequences was inferred by using the Maximum Likelihood method based on the Tamura-Nei model [19]. Initial tree for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value in the MEGA7 software suite. The isolates from Kashmir Himalayas separated into two clusters on phylogenetic tree (Fig. 2). The sheep isolates clustered with sheep ORFV strains reported from Greece and Italy. The two goat isolates and one sheep isolate (TVCC/Shuhama-02/Sheep) formed separate cluster with the goat ORFV strains reported from China. A retrospective history revealed that the sheep from which TVCC/Shuhama-02/Sheep strain was isolated had been cohabiting with the ORFV infected goats.

A significant observation from the VIR gene based phylogenetic analysis indicates that a host-specific genetic homogeneity exists among the ORFV strains. The ORFV strains isolated from sheep all around the globe formed distinct clusters, than that of the goat isolates. The same was observed among the ORFV strain isolated from Japanese serows (*Capricornis crispus*) which formed tight

Fig. 2 Phylogenetic analysis of ORFV based on nucleotide sequence of VIR gene. The analysis revealed that the ORFV strains from Kashmir Himalayas show species-specific genetic homogeneity, whereas as spatial genetic homogeneity was not strictly followed. Sheep-specific ORFV strain from Kashmir Himalayas clustered with ORFV strains isolated from sheep in Greece and Italy, whereas the goat-specific strains clustered with ORFV strains isolated from goats in China. Phylogenetic tree was created using on VIR gene sequences isolated in this study and the ORFV strain reported in GenBank (n = 72). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated. Each ORFV strain is identified by the country of isolation (CH: China, BZ: Brazil, GC: Greece, IT: Italy, JP: Japan, ML: Malaysia, NZ: New Zealand, SK: South Korea, UK: United Kingdom and US: United States), the isolate name as in public database, the species from which it was isolated and GenBank accession number in parenthesis

distinct cluster on the phylogenetic tree. The ORFV isolates from Kashmir also formed two separate clusters depending on the host-species from where they were isolated except TVCC/Shuhama-02/Sheep strain. However, the retrospective history suggested that the TVCC/Shuhama-02/Sheep strain may actually be a goat-specific strain which may have been caught by the sheep during cohabitation with the ORFV infected goats. The same may be true for the few other ORFV strains which clustered with the strains from non-homologous host on the phylogenetic tree. This observation conversely suggests that there may be an existence of a host-specific ORFV strain that has distinct predilection for a homologous host. A similar phenomenon has been observed in *Capripoxvirus* infection in sheep and goats, where a distinct host preference exists among Sheeppox virus and Goatpox virus strains [20]. In addition, the analysis revealed that the spatial genetic homogeneity was less strictly followed by the ORFV strains. The ORFV strains from a particular geographical entity were dispersed among different clusters on the phylogenetic tree. The sheep strains from Kashmir Himalayas clustered with the sheep strains from Greece and Italy, whereas the goat-specific strain clustered with the goat strains from China. The genetic closeness of the ORFV strains from Kashmir and China can be understood because of geographical contiguity; however, the closeness with the strains from Greece and Italy is difficult to comprehend. Nevertheless, in future the availability of more VIR gene sequences from the region and/or phylogenetic analysis based on the whole genome may shed more light on the species or spatial dynamics and evolution of the ORFV strains.

In summary, we described ORFV infection in the sheep and goats of Kashmir which was confirmed by the PCR based amplification of B2L gene in the scab samples. Based on the phylogenetic analysis of the VIR gene, distinct host-specific ORFV strains were found circulating in Kashmir that clustered together with sheep-specific

Chinese strains and goat-specific Greek/Italian strains. Taken together, this is first phylogenetic report of ORFV strains from the Kashmir Himalayas, and the data reported here will be useful for further research on the ORFV and its comprehensive prevention and control in the Kashmir Himalayas.

References

1. Bogoch II, Gandhi RT, Bibi Y, Dejsuphong V, Brown CM, Enis D, et al. Human orf virus infection from household exposures—United States, 2009–2011 (Reprinted from MMWR, vol 61, pg 245–248, 2012). *JAMA*. 2012;308(2):126–8.
2. Bora M, Bora DP, Barman NN, Borah B, Bora PL, Talukdar A, et al. Isolation and molecular characterization of orf virus from natural outbreaks in goats of Assam. *Virusdisease*. 2015;26(1–2):82–8. <https://doi.org/10.1007/s13337-015-0255-y>.
3. Bucch AS, Sudan NA, Willayat MM, Wani SA. A note on contagious ecthyma in exotic sheep. *Indian J Vet Med*. 1998;8(1):51–2.
4. Chan KW, Lin JW, Lee SH, Liao CJ, Tsai MC, Hsu WL, et al. Identification and phylogenetic analysis of orf virus from goats in Taiwan. *Virus Genes*. 2007;35(3):705–12. <https://doi.org/10.1007/s11262-007-0144-6>.
5. Dar KH, Tufani NA, Dar SH, Hafiz A, Naikoo M. Comparative therapeutic management of contagious ecthyma in small ruminants. *Intas Polivet*. 2015;16(2):431–5.
6. Hosamani M, Bhanuprakash V, Scagliarini A, Singh RK. Comparative sequence analysis of major envelope protein gene (B2L) of Indian orf viruses isolated from sheep and goats. *Vet Microbiol*. 2006;116(4):317–24. <https://doi.org/10.1016/j.vetmic.2006.04.028>.
7. Hosamani M, Scagliarini A, Bhanuprakash V, McInnes CJ, Singh RK. Orf: an update on current research and future perspectives. *Expert Rev anti-infect Ther*. 2009;7(7):879–93. <https://doi.org/10.1586/eri.09.64>.
8. Inoshima Y, Ito M, Ishiguro N. Spatial and temporal genetic homogeneity of orf viruses infecting Japanese serows (*Capri-cornis crispus*). *J Vet Med Sci*. 2010;72(6):701–7.
9. Kottaridi C, Nomikou K, Teodori L, Savini G, Lelli R, Markoulatos P, et al. Phylogenetic correlation of Greek and Italian orf virus isolates based on VIR gene. *Vet Microbiol*. 2006;116(4):310–6. <https://doi.org/10.1016/j.vetmic.2006.04.020>.
10. Kumar S, Stecher G, Tamura K. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol Biol Evol*. 2016;33(7):1870–4. <https://doi.org/10.1093/molbev/msw054>.
11. Kumar N, Wadhwa A, Chaubey KK, Singh SV, Gupta S, Sharma S, et al. Isolation and phylogenetic analysis of an orf virus from sheep in Makhdoom, India. *Virus Genes*. 2014;48(2):312–9. <https://doi.org/10.1007/s11262-013-1025-9>.
12. Li H, Zhu X, Zheng Y, Wang S, Liu Z, Dou Y, et al. Phylogenetic analysis of two Chinese orf virus isolates based on sequences of B2L and VIR genes. *Adv Virol*. 2013;158(7):1477–85. <https://doi.org/10.1007/s00705-013-1641-7>.
13. McInnes CJ, Wood AR, Mercer AA. Orf virus encodes a homolog of the vaccinia virus interferon-resistance gene E3L. *Virus Genes*. 1998;17(2):107–15.
14. Nitsche A, Buttner M, Wilhelm S, Pauli G, Meyer H. Real-time PCR detection of parapoxvirus DNA. *Clin Chem*. 2006;52(2):316–9. <https://doi.org/10.1373/clinchem.2005.060335>.

15. Peralta A, Robles C, Martinez A, Alvarez L, Valera A, Calamante G, et al. Identification and molecular characterization of orf virus in Argentina. *Virus Genes*. 2015;50(3):381–8. <https://doi.org/10.1007/s11262-015-1189-6>.
16. Rziha HJ, Bauer B, Adam KH, Rottgen M, Cottone R, Henkel M, et al. Relatedness and heterogeneity at the near-terminal end of the genome of a parapoxvirus bovis 1 strain (B177) compared with parapoxvirus ovis (orf virus). *J Gen Virol*. 2003;84(Pt 5):1111–6. <https://doi.org/10.1099/vir.0.18850-0>.
17. Spyrou V, Valiakos G. Orf virus infection in sheep or goats. *Vet Microbiol*. 2015;181(1–2):178–82. <https://doi.org/10.1016/j.vetmic.2015.08.010>.
18. Sullivan JT, Mercer AA, Fleming SB, Robinson AJ. Identification and characterization of an orf virus homologue of the vaccinia virus gene encoding the major envelope antigen p37K. *Virology*. 1994;202(2):968–73. <https://doi.org/10.1006/viro.1994.1420>.
19. Tamura K, Nei M. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol Biol Evol*. 1993;10(3):512–26.
20. Tulman ER, Afonso CL, Lu Z, Zsak L, Sur JH, Sandybaev NT, et al. The genomes of sheeppox and goatpox viruses. *J Virol*. 2002;76(12):6054–61.