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Phylogenetic analysis of Orf virus associated with contagious ecthyma (orf) outbreak in Tellicherry goats (*Capra hircus*)

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ABSTRACT

Orf virus (ORFV) is a member of genus *Parapoxvirus* that causes contagious ecthyma in goats. A pox-like disease was investigated in Tellicherry goats (31 female) maintained at a semi-organized farm. History revealed recent introduction of Tellicherry goats for breeding purpose and housing of the new entrants in to a farm already having a mild form of pox-like disease. Newly introduced and stressed Tellicherry goats developed severe form of infection with 100% morbidity. Affected goats showed lesions around lips (100%), commissure (53%) and oral cavity (65%); exanthematic dermatitis was evident in 94% of the affected goats followed by ulceration (47%) and nodular lesions (24%). Scab samples were collected from affected goats to confirm the clinical diagnosis. Genus *Parapoxvirus* was confirmed by the amplification of specific 594 bp and 235 bp amplicons. Further, Orf virus specific amplicon of size 1,206 bp was also amplified for the confirmation. Sequence analysis of PCR amplicons showed close resemblance of the outbreak strain with reported Indian Orf virus isolates. Based on the homology of the outer envelope protein B2L gene sequence of Orf virus, the source of infection to the Tellicherry goats was traced to the local goat. Although Orf virus is zoonotic; however, no occupational transmission was noticed in the present outbreak.

Key words: B2L gene, Goat, Karnataka, Orf virus, Parapoxvirus, Sequence analysis

Contagious ecthyma (also known as scabby mouth, ecthyma contagiosum, contagious pustular dermatitis or Orf) is an epitheliotrophic skin infection of mostly ruminants (Zhang *et al*. 2014). Orf virus is known to infect humans and cause painful pustular lesions over hands and fingers (Al-Salam *et al*. 2008). Orf usually occurs as an outbreak in small ruminants like goat and sheep populations (Mondal *et al*. 2006); and the disease has been reported from several countries like India, Korea, China, Brazil, Turkey, Finland, Germany, Norway, Japan, Italy, United Kingdom, Australia and several Asian, American or European countries (Hosamani *et al*. 2006, Oem *et al*. 2009, Chan *et al*. 2007, Abrahao *et al*. 2009, Zhang *et al*. 2010, Zhao *et al*. 2010, Li *et al*. 2012, Karakas *et al*. 2013). Human infections mostly occur due to the occupational exposure (Carr 1968, Paiba *et al*. 1999).

Orf is caused by Orf virus, a prototype virus of genus *Parapoxvirus* belonging to sub-family Chordopoxvirinae of family Poxviridae. Orf virus has a double stranded GC rich (63.5%) DNA of size 134–139 kb (Wittek *et al*. 1979, Chan *et al*. 2009, Hosamani *et al*. 2009, Mahmud *et al*. 2014) and the genus *Parapoxirus* also includes Bovine Papular Stomatitis virus (BPSV), Pseudocowpox virus (PCPV) and Parapoxvirus of red deer of the New Zealand (PVNZ) (Esposito *et al.* 1995). These epitheliotrophic DNA viruses can be identified by amplifying viral DNA using polymerase chain reaction in clinical sample obtained from outbreaks. Using specific primers Parapoxvirus viruses can be discriminated *viz*. Vaccinia and Fowlpox viruses (Inoshima *et al*. 2000). The B2L gene of Parapoxviruses encodes a highly immunogenic envelope protein p42K (Sullivan *et al*. 1994) which is a major envelope protein of Orf virus; it has an open reading frame of 1,137 nucleotides and encode a polypeptide of 378 amino acids (Hosamani *et al*. 2006). The B2L gene is one of the most widely used target for establishing molecular phylogeny of the virus. Present investigation was undertaken to confirm the pox-like disease outbreak occurred in a flock of goats using PCR and to establish molecular phylogeny of Orf virus based on major envelope gene (B2L) sequence analysis. Nucleotide sequences of the Orf virus outbreak were compared with reported Orf viruses of goats so as to establish phylogenetic relationships.

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MATERIALS AND METHODS

History of outbreak: An outbreak of pox-like disease was observed among 31 female, one year old Tellicherry breed of goat maintained at a semi-organized farm (M.C. Halli village, Tarikere Taluka, Chikmagalur District, Karnataka, India; coordinates 13°45'57.8"N and 75°46'03.3"E) during July 2015. History revealed recent introduction of Tellicherry goats from the neighbouring Kerala state for breed up-gradation purpose into a goat farm that was already occupied by 110 local goats. In the preceding 30 days, local goats had suffered from a mild pox-like disease and had eventually recovered without any complications or deaths.

Risk factor for the outbreak: Transport induced stress and immuno-suppression was the major factor for the Orf outbreak among the goats. Goats were transported from the Kerala to Karnataka (about 500 km distance). Upon arrival at the farm, Tellicherry goats appeared healthy without any lesion (parent farm in Kerala did not have any history of such infection). However, when housed in the vicinity (10 feet distance) of the previously mildly infected local goats, the healthy Tellicherry goats also developed active and severe disease in a span of 30 days.

Recording of clinical manifestations and collection of sample: In the present study, individual goats were thoroughly examined by a veterinarian and data was gathered through a semi-structured questionnaire indicating location, nature and severity of lesions in the affected goats. Scab samples were collected from all the affected goats (Tellicherry as well as local) and transported to the laboratory for further analysis.

DNA extraction, PCR and sequencing: Scab samples collected from Tellicherry and local goats were subjected for DNA extraction. Briefly, scab samples were triturated in phosphate buffered saline and freeze-thawed several times followed by DNA isolation using a commercial kit (Hi-Media, Mumbai). For the confirmation of genus *Parapoxvirus*, initial PCR amplification was performed using PPP-1 + PPP-4 primers followed by a semi-nested PCR using PPP-3 + PPP-4 primers (Table 1) as reported by Inoshima *et al*. (2000). Orf virus was confirmed using specific primers OVB2LF1 + OVB2LR1 as described by Hosamani *et al*. (2006). Ready-to-use master mix (Hi-Media, Mumbai) was mixed with the template DNA sample (5 µl) along with primers (10 picomoles) and PCR was performed in thermal cycler following the conditions mentioned by the authors. The PCR products were electrophoresed (1.0% agarose gel), visualized under gel documentation system and amplicons were custom sequenced (Chromos Biotech, Bengaluru). Orf virus sequences associated with outbreak were analyzed by BLAST tool (http://www.ncbi.nlm.nih.gov/blast) of NCBI and nucleotide sequences of B2L gene were downloaded from the GenBank (NCBI). Multiple sequence alignment was carried out using Clustal W method of Meg-Align tool of LaserGene, DNA Star (Clewley 1995, Burland 2000) and dendrogram was constructed.

RESULTS AND DISCUSSION

An outbreak of contagious ecthyma was observed in goat flock; Tellicherry breed of goats acquired infection after their introduction into a farm having local goat that suffered Orf. Transportation induced stress was construed as the cause of the outbreak. Tellicherry goats (31) (one year age) showed severe form of skin infections with 100% morbidity without any mortality. Orf associated mortality in small ruminants may be very high due to secondary (bacterial or fungal) infections (Haig and Mercer 1998, Robinson 1983, Zhang *et al*. 2010). However, owing to the timely management of the cases no such mortality was observed in the present episode.

Clinical manifestations: Severity of clinical signs was highest on 10th day of infection. Affected goats (65% well-built and 35% weak) were found active but refused feed due to lesions in and around the mouth, lips (100%), commissure (53%) and oral cavity (65%). Exanthematic

Table 1. Oligonucleotide primer sequences used for PCR amplification of Orf virus

Primer	Sequence	Thermal cycling conditions	Size	Reference
PPP-1	5'-GTCGTCCACGATGAGCAGCT-3'	1) 95°C, 9 min (1 cycle)	594 bp	Inoshima
PPP-4	5'-TACGTGGGAAGCGCCTCGCT-3'	2) 94°C, 1 min; 55°C, 1 min and 72°C, 1 min (30 cycles)		*et al.* (2000)
		3) 72°C, 7 min (1 cycle)		
PPP-3	5'-GCGAGTCCGAGAAGAATACG-3'	1) 95°C, 9 min (1 cycle)	235 bp	
PPP-4	5'-TACGTGGGAAGCGCCTCGCT-3'	2) 94°C, 1 min; 50°C, 1 min and 72°C, 1 min (5 cycles)		
		3) 94°C, 1 min; 55°C, 1 min and 72°C, 1 min (25 cycles)		
		4) 72°C, 7 min (1 cycle)		
OVB2LF1	'5-TCCCTGAAGCCCTATTATTTTTGTG-3'	1) 94°C, 3 min (1 cycle)	1,206 bp	Hosamani
OVB2LR1	'5-GCTTGCGGGCGTTCGGACCTTC-3'	2) 94°C, 1 min; 52°C, 1 min and 72°C, 1 min (29 cycles)		*et al.* (2006)
		3) 72°C, 7 min (1 cycle)		

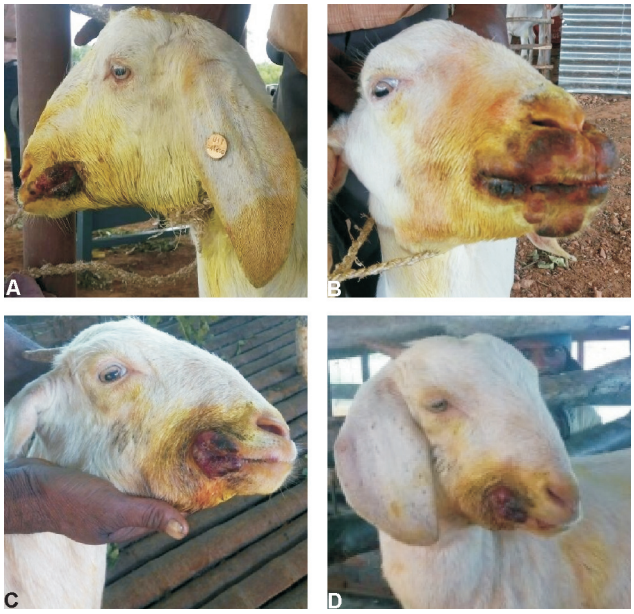

Fig. 1. Orfvirus lesions in Tellichery breed of goat (A, Proliferative; B, Nodular; C, Exanthematous and D, Ulcerative).

dermatitis was evident in 94% of the affected goats followed by ulceration (47%) and nodular lesions (24%) (Fig. 1). About 88% goats showed secondary bacterial infections as evidenced by pus in the lesions. After its peak on 10th day, clinical signs of the disease subsided with administration of antibiotic therapy to combat secondary bacterial infections.

Orf is a non-systemic viral infection caused by Orf virus and infection is mostly characterized by pustular lesions around mouth and nostrils. Similar clinical observations were also made by Hawkins *et al*. (1991) and Bora *et al*. (2015) in goats. Mazur and Machado (1989) reported 100% morbidity and 93% mortality in goats due to Orf.

Orf virus is known to infect humans like other zoonotic parapoxviruses such as bovine popular stomatitis virus and pseudocowpox virus. Orf virus causes cutaneous lesions in humans mostly in occupationally exposed individuals; lesions are characterized by large, painful nodules over hands and less frequently on the face (Bowman *et al*. 1981, Meechan and MacLeod 1992, Sanchez *et al*. 1985, Lewis-Jones 2004). Outbreaks of Orf virus in goats have led to zoonotic infections in humans previously (Al-Salam *et al*. 2008, Malik *et al*. 2009, Georgiades *et al*. 2005). Nevertheless, none of the occupationally exposed human acquired Orf infection in the present episode.

Molecular characterization: Pox-like disease was noticed in a flock of goats and the infection was confirmed as Orf based on the amplification of specific genes (genus *Parapox* and Orf virus) using diagnostic PCR (Fig. 2). Nucleotide sequence of Orf virus was analyzed to establish phylogenetic relation between outbreak strain with other reported stains (Fig. 3; Tables 2, 3). Genus *Parapoxvirus* was confirmed based on the PCR amplification of 594 bp (PPP1 and PPP4 primers) and semi-nested PCR amplicon of 235 bp (PPP3 and PPP4 primers) (Fig. 2 A,B). Outbreak virus was identified as Orf virus by amplifying 1,206 bp major envelope protein gene of the Orf virus (primers OVB2LF1 and OVB2LR1) as shown in Fig. 2C. Based on the history and PCR amplification of specific genes using scab samples, transmission of Orf virus from the local goat to the newly introduced Tellicherry breed of goats was established.

Sequence analysis and phylogeny: Nucleotide alignment using BLAST tool of NCBI of B2L gene of Orf virus of

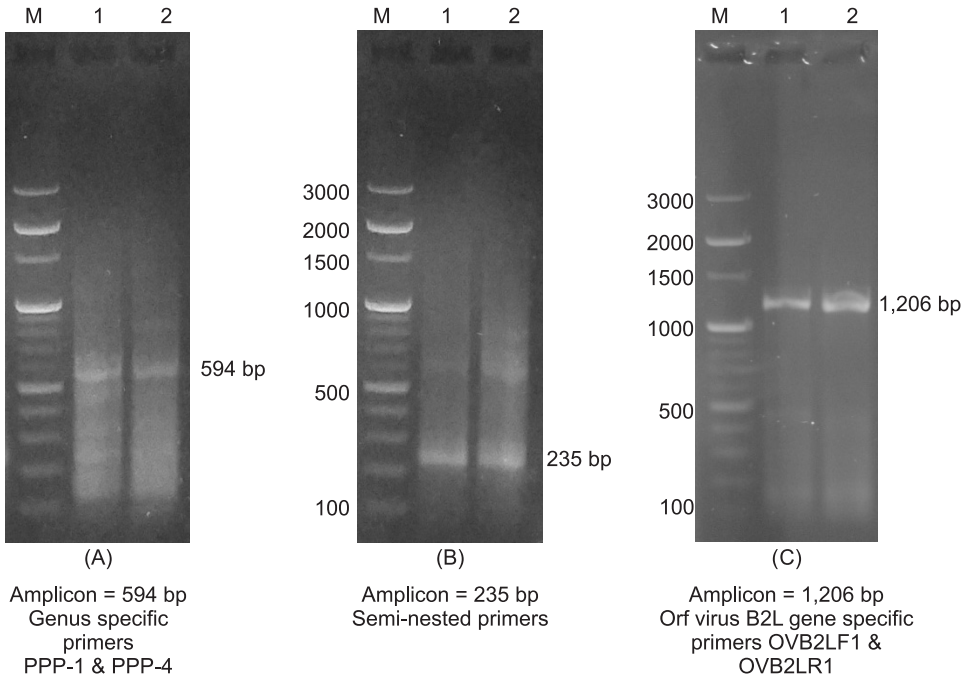

Fig. 2. Ethidium bromide stained agarose gel images of PCR products. (Lane 1, Local goat; Lane 2, Tellichery goat and Lane M, DNA Marker, 100 bp).

both local and Tellicherry goats showed highest homology with Basudebpara/Tripura/14 (GenBank Accesion No. KT935590.1) followed by South Chandrapur/Tripura/2014 (KX377974.1) and Baulia Basti/Tripura/14 (KT935589.1) isolates. Orf virus sequences of both goats had 100% homology indicating its transmission from local goats to Tellicherry goats. Outbreak strain was phylogenetically related to other reported Indian Orf viruses (Tables 2, 3; Fig. 3). Sequence homology and closer phylogenetic relationship between Orf virus strains of Karnataka and Tripura region of India indicates widespread dissemination of the Orf viruses across the country.

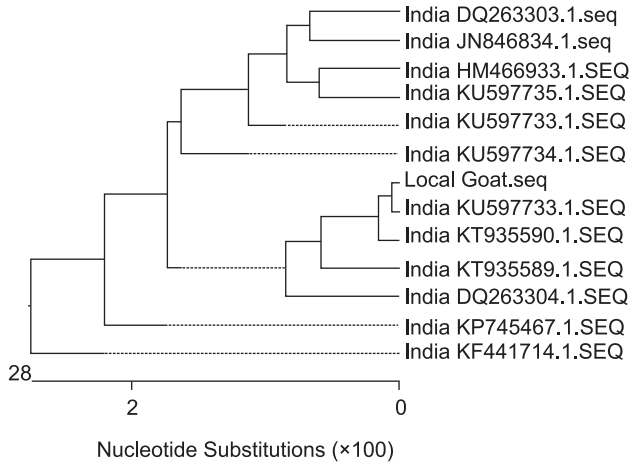

Fig. 3. Phylogenetic relationship of outbreak strain with other Orf virus strains (aligned by Clustal W of MegAlign software).

Table 2. Sequences of major envelope protein (B2L) gene of goat Orf virus (ORFV) of Indian strains used for phylogenic analysis

Strain (NCBI Accession Number)
Muk/2000 (HM466933); India 82/04 (DQ263303); 59/05 (DQ263304); Assam/09 (JN846834); Chitradurgar/Goat-19 (KU597733); Chitradurgar/Goat-20 (KU597734); Chitradurgar/Goat-36 (KU597735); CPD/IND2013/02 (KF441714); CIRG/2014 (KP745467); Basudebpara/Tripura/14 (KT935590); Baulia Basti/Tripura/14 (KT935589)

In a similar study based on B2L gene phylogenetic analysis, Bora *et al*. (2015) reported close relationship between outbreak strain of Orf viruses of the North Eastern region of India. Orf viruses have shown increased emergence into new territories and species (Hosamani *et al*. 2009, Maganga *et al*. 2016). Phylogenetic analysis of B2L and F1L genes showed close homology between the Malaysian strain with that of Chinese and Indian Orf virus isolates (Abdullah *et al*. 2015). Based on the B2L gene phylogeny, Maganga *et al*. (2016) showed common Asian ancestral origin of South Korean and Gabonese strains of Orf virus. Similarly, El-Tholoth *et al*. (2015) also established closer relationship between the Egyptian Orf virus with the Israel Orf viruses. Sequence analysis of A32L and B2L genes of Orf virus isolates indicated two major clusters responsible for outbreaks in Ethiopia (Gelaye *et al*. 2016). However, with the existing data, no such conclusion could be made for Indian orf virus isolates.

In the present study, the B2L gene analysis of orf virus of the local goat (non-discript type) and its 100% homology with the orf virus of the newly introduced Tellicherry breed goat indicated aquisition of severe form of Orf infection by the later consequent to the transportation stress.

Orf virus occurs worldwide (Mondal *et al*. 2006) with its incidence reaching to 90% in small ruminants and mostly young animals suffer from a severe form of disease (Haig and McInnes 2002). Orf is an infection of economic significance, loss of young ones or weight loss affects economy or livelihood of farmers in low economic countries.

Laboratory diagnosis of Orf virus includes viral isolation, electron microscopy, counter-immuno electrophoresis, serum neutralization tests, enzyme assays, and PCRs. However; each method has its own advantage and limitations. Recently, real time PCR detection based TaqMan probe (Gallina *et al*. 2006) and chimeric fluorescent dyes such as SYBR Green I (Wang *et al*. 2017) have been developed for high throughput detection of the virus.

Table 3. Partial B2L gene sequences homology between the Orf virus strains aligned using Clustal W method of MegAlign program (Lasergene, DNASTAR)

Percent identity

	1	2	3	4	5	6	7	8	9	10	11	12	13	
1		98.0	98.0	97.5	97.9	56.0	98.2	99.0	99.7	35.7	98.2	41.0	100.0	1
2	2.0		97.5	97.9	98.7	58.7	88.8	98.4	98.2	48.1	95.2	51.3	73.7	2
3	2.0	2.5		98.2	97.7	59.5	89.6	98.4	98.3	47.8	95.6	51.0	74.0	3
4	2.6	2.1	1.9		98.3	59.2	89.4	97.7	98.0	48.3	95.2	51.5	73.4	4
5	2.2	1.3	2.3	1.7		59.3	89.4	98.2	98.4	48.3	95.6	51.5	73.6	5
6	2.5	3.9	2.5	3.0	2.8		91.3	97.4	97.7	76.8	98.1	82.5	64.4	6
7	1.9	2.8	1.9	2.2	2.1	1.6		98.3	98.2	46.9	97.9	50.6	80.3	7
8	1.0	1.6	1.6	2.3	1.9	2.6	1.8		98.7	47.6	95.5	50.9	74.5	8
9	0.3	1.9	1.7	2.1	1.6	2.3	1.9	1.3		47.9	96.0	51.1	75.0	9
10	2.8	1.6	2.2	1.3	1.3	2.6	1.8	2.8	2.0		95.8	96.1	53.0	10
11	1.9	2.2	1.7	2.2	1.7	1.9	2.2	1.8	1.4	1.3		52.4	76.1	11
12	2.7	1.7	2.2	1.2	1.2	2.3	1.9	2.4	2.1	1.5	1.7		57.2	12
13	0.0	2.0	1.7	2.4	2.1	2.2	2.0	0.9	0.2	2.6	1.5	2.6		13
	1	2	3	4	5	6	7	8	9	10	11	12	13	

Divergence

Local Goat.seq
India DQ263303.1.seq
India DQ263304.1.SEQ
India DQ263304.1.SEQ
India HM466933.1.SEQ
India JN846834.1.seq
India KF441714.1.SEQ
India KP745467.1.SEQ
India KT935589.1.SEQ
India KT935590.1.SEQ
India KU597733.1.SEQ
India KU597734.1.SEQ
India KU597735.1.SEQ
Tellicherry.seq

Isothermal amplification based assays for the rapid visual detection of Orf virus have also been developed (Yang *et al*. 2015, 2016).

Orf virus is environmentally stable and it is a highly contagious pathogen. In India, contagious ecthyma is endemic; shared pasture lands, lack of biosecurity at farms, poor husbandry practices, higher stocking density and increased contact between the affected and susceptible animals act as risk factors leading to outbreaks (Hosamani *et al*. 2006, Mondal *et al*. 2006). Keeping in view the economic impact on farmers, animal health and zoonotic potential of the Orf virus, further studies are needed to elucidate the epidemiological pattern and spread of Orf viruses across the wide geographical areas.

Cutaneous pox-like lesions were noticed in Tellicherry goats soon after their introduction into a farm already having local goats with similar mild disease. Transportation stress induced severe form (100% morbidity) of infection was identified as Orf virus infection based on the amplification of genus and virus specific genes. The phylogenetic relationship between the Orf virus responsible for the outbreak in goats and other Indian Orf viruses was established.

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