Occurrence and identification of contagious ecthyma in blackbuck

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Abstract A carcass of male free ranging adult blackbuck (*Antelope cervicapra*) was presented for necropsy examination exhibiting thick confluent nodular skin lesions around the mouth and the dry scaly crusts/fissures on the skin of abdomen, thigh and shoulder with subcutaneous haemorrhages. The skin sample around mouth was found positive for orf virus (ORFV) identified by counterimmunoelectrophoresis and PCR. Histopathology of the mouth skin revealed the hyperkeratinization, epidermal sloughing and epithelial hyperplasia showing acanthosis with rete ridges and few eosinophilic intracytoplasmic inclusion bodies in keratinocytes. Further, comparative *B2L* gene sequence analysis revealed that the virus isolate from blackbuck had shown 97.8–99.6 and 97.6–99.5 % identity at nucleotide and amino acid levels respectively with Indian isolates and maximum identity with ORFV 79/04, an isolate from India. Phylogenetic analysis based on *B2L* gene also revealed the same evolutionary relationship that it is closely related to Indian isolates. This seems to be the first report of orf in blackbuck from Indian subcontinent.

Keywords Blackbuck · Mange · Contagious ecthyma · *B2L* gene · Phylogeny

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Contagious ecthyma (CE) also known as sore mouth or scabby mouth or orf is a highly contagious, zoonotic viral disease caused by orf virus (ORFV) of the genus *parapox virus* of the family *Poxviridae* [3, 7]. It affects sheep, goats and other domesticated/wild ruminants namely rein deer, musk ox, mule deer, white-tailed deer, pronghorn fawns and wapiti calves in natural captive/free ranging conditions as well as experimental infection [2, 9, 16]. It has also been reported in camelids, squirrels and seals [4]. Secondary bacterial and fungal infections are common [2] and sometimes associated with other viral infections namely PPRV [13] and goat pox [17]. Increasing number of reports of CE in sheep and goats has been noticed worldwide and it has been reported in all parts of India and endemic in nature [4, 10]. This endemic disease incurs economic losses by causing severe morbidity in adults and mortality in young ones affecting farming community in India [10]. Secondary bacterial infection of orf lesions involving *Staphylococcus aureus* and *Arcanobacterium pyogenes* and severity aggravated by associated myiasis have been reported during orf cases [5]. There have also been reports of lambs with dual orf/papilloma virus infection and orf/sheep pox virus infection [19, 20]. Enhanced severity of orf lesions and other complications such as mastitis in ewes and foot rot have been noticed in secondary bacterial and associated fungal infections [10]. Outbreaks of CE in wild animal species from captive or free ranging or a zoological collection could be of considerable significance in virus perpetuation or spill over to nearby domestic small ruminants. Furthermore, the role of wildlife in the epizootiology of orf has not been fully elucidated. In India, neither ORFV sero-prevalence/infections have been reported often nor were systematic attempts on virus antigen identification and genomic characterization made for wild animals in past. In this study, an investigation of CE infected free

range blackbuck associated with sarcoptic mange is reported. Sarcoptic mange is a highly contagious parasitic infection caused by a mite (*Sarcoptes scabiei*) that burrows into the epidermis of animals and reported in domestic, free ranging and domestic wild mammals [9]. This investigation is the first report of CE in the free-ranging blackbuck that found dead with severe skin lesions and presented for necropsy examination at wildlife section of the institute. The ORFV isolate from blackbuck was confirmed for specific antigen and viral nucleic acid respectively by counterimmunoelectrophoresis (CIEP) and *DNA polymerase* gene based diagnostic PCR followed by full length *B2L* gene sequencing and phylogenetic analysis. This study on CE in blackbuck could create awareness on the epidemiology and possible transmission of ORFV by wild ruminants to domestic animals and vice versa.

A carcass of male blackbuck was found dead exhibiting severe skin lesions in social forestry, Division, Bareilly, Uttar Pradesh, India. The carcass was presented for necropsy examination at wildlife section, Indian Veterinary Research Institute, Izatnagar. The salient gross lesions were noted and morbid samples from different tissues were collected for laboratory investigations. Briefly, the tissue samples from skin and other visceral organs were collected in 10 % neutral buffered formalin and processed for histopathological examinations as per standard technique. The scrapings from the skin lesions of different body parts were collected in 10 % potassium hydroxide (KOH) and processed for parasitological examination. For virological investigation, skin scab sample especially around mouth was processed as 10 % suspension using sterile phosphate buffered saline (0.1 M) and was used in counter immune electrophoresis (CIE) to identify ORFV antigen and total genomic DNA (gDNA) extraction for PCR/cloning and for virus isolation in primary lamb testes cells after repeated freezing and thawing as per standard protocols [18].

Initially semi-nested PCR [6] followed by diagnostic PCR [17] and *B2L* gene PCR [18] were conducted. *B2L* PCR product was sequenced after cloning into pGEMT-Easy vector (Promega, Madison, USA) for further confirmation. Edited complete *B2L* sequence (with open reading frame of 1137 bp) of blackbuck was aligned by MegAlign program (DNA STAR, Lasergene 6.1) for identity at nucleotide (nt) and deduced amino acid (aa) levels by comparing with a total of fifty one ($n = 51$) gene sequences of different parapox viruses from GenBank database. Phylogenetic tree based on deduced amino acid sequence of *B2L* was also constructed by using bootstrap test of phylogeny in the neighbor joining method in MEGA 5.1 software [15]. Scab suspension along with antibiotics was inoculated in primary or secondary lamb testes cells grown in Eagle's minimum essential medium supplemented with 10 % new born calf serum and maintained in EMEM with 2 % serum as described earlier [18]. CIE test was performed to identify ORFV antigen using reference ORFV anti-serum available in author's laboratory [14].

Necropsy findings of the carcass include poor body condition, dehydration, dried, firm, crusty and fissured skin coat on abdomen, thigh and shoulder regions and thick confluent nodular skin lesions around the mouth (Fig. 1a). Histopathology of the mouth skin showed epithelial hyperplasia, hyperkeratinization, with increased thickness up to 5–10 times normal (acanthosis) with anastomosing rete ridges extended deep into the dermis (Fig. 1b). Some of the keratinocytes lining the ridges showed presence of single or multiple variable sized eosinophilic intracytoplasmic inclusion bodies (Fig. 1b, inset). The skin scrapings and sections of the skin covering the parts other than mouth showed presence of *Sarcoptes* mite in the superficial epidermal layers. The skin lesion from mouth was found positive for ORFV antigen in CIEP test. The semi-nested PCR resulted in 235 bp product as expected [6] from

Fig. 1 **a** Blackbuck male-thick nodular skin lesions around the mouth. **b** Epithelial hyperplasia with increased thickness up to 5–10 times normal (acanthosis). H&E, $\times 40$ (inset Fig (arrow): keratinocytes contained one or more round, brightly eosinophilic intracytoplasmic inclusion bodies. H&E, $\times 400$)

Fig. 2 **a** Diagnostic PCR showing amplification of 214 bp product of DNA polymerase gene of ORFV in blackbuck sample. Lane M: 100 bp ladder plus DNA marker; Lane 1 ORFV Mukteswar 59/05 as positive control; Lane 2 blackbuck skin scab; Lane 3 Vero cell DNA; Lane 4 NTC, **b** PCR amplification of *B2L* gene of ORFV from blackbuck skin lesion. Lane M 100 bp ladder plus DNA marker; Lane 1 blackbuck sample; Lane 2–3 ORFV Mukteswar 59/05 and ORFV

Muk/09 and Lane 4 NTC, **c** Phylogenetic analysis of blackbuck isolate with different parapoxviruses based on complete *B2L* coding sequence. Unrooted tree was constructed using neighbour-joining method of bootstrap test of phylogeny in the MEGA version 5.1 program. Numbers on the tree branches represent the bootstrap support calculated per 1000 bootstrap replicates. The scale bar beneath the tree represents the amino acid substitutions per site

blackbuck sample. Further, the etiology was confirmed by diagnostic PCR and *B2L* gene PCR showing the amplification of 214 bp (Fig. 2a) and 1206 bp (Fig. 2b) products respectively as described earlier [17, 18]. The results of CIEP and *B2L*/DNA Polymerase gene based PCR assays confirmed the infection of the animal by ORFV. However, we could not isolate the virus from sample even after six times of blind passages in PLT/SLT cells.

Sequence analysis of the *B2L* coding gene sequence of blackbuck/Bareilly/14 (# KT191487) shares a 97.6–99.6 and 97.6–99.5 % identity with Indian ORFV isolates with a maximum identity (99.6 and 99.5 %) to sheep isolate from Mukteswar (ORFV 79/04, #DQ263306) at nt and aa levels, respectively. It also shares about 98.2 and 97.9 % identity with Indian vaccine strain (ORFV Muk 59/05), respectively at nt and aa levels. Besides, a percent identity of 83.8–83.9 and 83.4 with BPSV isolates whereas, 93.6–95.1 and 94.7–95 with PCPV was observed, at nt and aa levels, respectively. It shares a significant identity of 97.8–98.2 and 97.4–98.4 % with ORFV isolates worldwide for which the complete genome has been sequenced and

characterized (IA82, SA00, NZ2 and D1701 isolates) as observed in previous reports [1, 18]. Among Asian isolates, ORFV JS/04 of China shared maximum percent identity of 99.4 and 99.2 with this isolate under the study. In addition to above, blackbuck isolate shared a 93.3–93.5 and 94.7–95 % identity with camel CE isolates (Jodhpur/09 and K2-SD) at nt and aa levels respectively. A total of six synonymous (A63C, C234A, G267A, C453A, G767A, A930G) and three non-synonymous mutations (G553A, A733G and G880A) leading to three amino acid changes (V185I, I245 V and D294 N) have been noticed in multiple sequence alignment of blackbuck isolate under this study. These three amino acid changes are found to be unique for Indian isolates as observed/reported elsewhere [18]. The overall percentage of nt and aa identity of blackbuck isolate with ORFV isolates and other parapoxviruses are presented in Table 1.

Phylogenetic analysis revealed that all the members of PPVs clustered into three distinct groups (ORFV, PCPV and BPSV) and the isolate under the study is found grouped with Indian ORFV isolates as monophyletic and

Table 1 Percent identities of *B2L* coding sequences of blackbuck isolate from Bareilly, India with other members of the genus *Papapoxvirus*

ORFV and other PPV isolates	ORFV blackbuck/Bareilly/14	
	Nucleotide	Amino acid
Indian ORFVs	97.8–99.6	97.6–99.5
ORFV Ind/Muk 59/05 (Vaccine)	98.2	97.9
Asian ORFVs	97.5–99.4	96.6–99.2
ORFV SA/00	98.0	97.4
ORFV IA82	98.3	97.4
ORFV D1701	97.8	97.6
ORFV NZ2	98.2	98.4
Other ORFVs worldwide	97.4–99.0	96.3–98.9
ORFV musk ox	97.8	96.8
ORFV Camel isolates	93.3–93.5	94.7–95.0
PCPV isolates	93.6–95.1	94.7–96.2
BPSV isolates	83.8–83.9	83.4

closely related to ORFV Ind 79/04, an isolate of sheep from Mukteswar, India (Fig. 2c). Infected PLT cells were daily observed for appearance of virus-induced cytopathic changes. As there was no CPE changes at passage level 1, passaging of infected cells was continued for 6 passages in primary lamb testes and checked in diagnostic PCR for ORFV DNA. However, ORFV could not be recovered from blackbuck sample and it might be due to loss of intactness of viral envelope proteins of ORFV during storage and delay in transport of morbid materials to virological investigation. However, the presence of viral nucleic acid in skin tissue was identified by molecular diagnostics namely diagnostic PCR and *B2L* gene cloning/sequencing. Multiple sequence alignment and phylogenetic tree based on complete *B2L* sequence have been performed to study the evolutionary relationship with other circulating virus strains/isolates in the country [1, 4, 18].

The source of infection of this case was unknown, although there was a possibility of transmission of virus from diseased sheep and goats at the border areas of forest range. The blackbuck was in free range to graze in the pastures of cattle and sheep near the forest range. Since the infectivity of parapoxvirus under natural conditions is extremely stable [10], it is likely that blackbuck have been exposed to the virus. It has been reported that CE is endemic and reported often in Indian small ruminants even after first exposure [10, 15] and the present study also showed the ORFV from blackbuck is closely related with previous Indian isolates (ORFV 67/04, ORFV 79/04 and ORFV Muk/2000) indicating the circulating Indian ORFV strains spread to blackbuck. The severe mange infection of blackbuck could have allowed the ORFV to enter into the

animal as the virus is opportunistic pathogen as reported earlier for mixed infection of goat with PPRV and ORFV [13]. The histopathological lesions were not very severe as compared to those reported in sheep and goats affected with ORFV [8], except lesions like epithelial hyperplasia, acanthosis and presence of eosinophilic intra-cytoplasmic inclusion bodies in the epidermal cells of mouth skin. Cases of CE are common in sheep and goat and wild ruminants [3, 7] but it is rarely reported in Antelope family. It was reported experimentally in mule deer fawns, white-tailed deer fawns, pronghorn fawns, wapiti calves and natural cases in rein deer [2, 9, 16]. The present case showed mixed infection of orf with sarcoptic mange infection, however the mange is common in wild ruminants [12] that could have attracted the ORFV, an opportunistic pathogen either by direct contact with contaminated pasture or through fomites. Earlier a case of CE with caseous lymphadenitis has been reported [11]. However it has not been reported with mange infection earlier. It seems that there may be an initial infection of sarcoptes mite followed by entry of ORFV through damaged skin to cause CE as secondary infection or the animal might have contracted the infection simultaneously.

In conclusion, case history, gross lesions, histopathology and various virological diagnostic investigations identified the etiology of the case of blackbuck as CE. The starvation due to sore mouth evidenced by empty stomach on examination caused the death of animal. Furthermore, sequence and phylogenetic analyses of the *B2L* gene revealed the genetic relationship with other ORFV isolates circulating in India. To the best of the authors' knowledge, this appears to be the first report of detection and genetic characterization of ORFV from blackbuck in Indian sub-continent. Therefore, emphasis should be given in future for continuous serological/clinical surveillance of ORFV in wild ruminants to determine the prevalence, its impact on wildlife conservation and the possible role of these species in the transmission of ORFV.

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