

Sequence and phylogenetic analyses of an Indian isolate of orf virus from sheep

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Summary

The authors describe the isolation and identification of orf virus (ORFV) from an outbreak in a flock of sheep at Mukteswar, Uttarakhand, India, in 2009. The causative agent, ORFV was successfully isolated in primary lamb testes cells and identified using a semi-nested diagnostic polymerase chain reaction (PCR) and sequence and phylogenetic analyses of immunogenic envelope protein (B2L) coding gene. The affected animals showed characteristic proliferative skin lesions around the mouth and on nostrils and, in a few animals, lesions were also noticed on the tongue irrespective of age and sex. The morbidity, mortality and case fatality rates observed were 6%, 45% and 13%, respectively. Clinical samples were initially screened by counter immuno-electrophoresis and the serum neutralisation test; further positive skin scabs were tested with diagnostic PCR and virus isolation was performed on primary or secondary lamb testes cultures. Sequencing and phylogenetic analyses of the sheep isolate based on the B2L gene revealed that the isolate was closest to a goat isolate retrieved from an outbreak at the same geographic location in 2000. Furthermore, it also showed close genetic similarities with other Indian isolates reported earlier. Regular and systematic investigation of outbreaks is necessary to monitor the disease in susceptible populations. The development of rapid diagnostic methods as well as

effective vaccine to control this infection not only from India but also other parts of the world is called for.

Keywords

Contagious ecthyma, Epidemiology, India, Orf, Parapoxvirus, Phylogenetic analysis, Polymerase chain reaction, Sequence, Virus.

Analisi sequenziale e filogenetica di un isolato indiano di *Parapoxvirus ovis*

Riassunto

Gli autori descrivono la modalità di isolamento e identificazione del Parapoxvirus ovis da un focolaio che ha colpito, nel 2009, un gregge di pecore nella zona di Mukteswar, Uttarakhand, India. L'agente patogeno, il Parapoxvirus ovis (ORFV), è stato isolato in cellule primarie (testicolo di agnello) e identificato mediante semi-nested PCR (sn-PCR) diagnostica e analisi sequenziali e filogenetiche del gene codificante la proteina envelope immunogenica (B2L). Gli animali contagiati hanno mostrato le tipiche lesioni cutanee proliferative nell'area intorno a bocca e narici. In alcuni casi sono risultate presenti lesioni anche sulla lingua, indipendentemente dall'età e dal sesso. I tassi di morbilità, mortalità e letalità sono risultati rispettivamente pari a 6%, 45% e 13%. I campioni clinici sono stati inizialmente esaminati con counter immunoelettroforesi e test di neutralizzazione sierica. Altre croste positive sono state

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sottoposte a PCR diagnostica e il virus è stato isolato in colture cellulari primarie o secondarie (testicoli di agnelli). Le analisi sequenziali e filogenetiche dell'isolato, basate sul gene B2L, hanno evidenziato come l'isolato fosse estremamente simile a quello di una capra contagiata durante un focolaio verificatosi, nell'anno 2000, nella stessa area geografica. L'isolato ha mostrato una relazione genetica con altri isolati indiani oggetto di precedenti analisi. Per quanto riportato si evidenzia la necessità di indagini regolari e sistematiche dei focolai per monitorare la malattia nelle popolazioni vulnerabili. Si auspica altresì lo sviluppo di rapidi metodi diagnostici e la vaccinazione effettiva delle greggi per tenere sotto controllo l'infezione non solo in India ma anche in altre regioni del mondo.

Parole chiave

Ectima contagioso, Epidemiologia, Filogenesi, India, Orf, *Parapoxvirus ovis*, Reazione a catena della polimerasi, Virus.

Introduction

Parapoxviruses (PPV) are epitheliotrophic and affect a wide range of animals, including wild species and humans (12, 13). Among these, the orf virus (ORFV) is an opportunistic pathogen that is often associated with other viral infections, namely: capripox and peste des petits ruminants virus infections (20). The virus is the prototypic member of the genus *Parapoxvirus* and belongs to the family *Poxviridae* and is a double-stranded DNA (dsDNA) virus, like other pox viruses (6). Other important members of the genus are pseudocowpox virus (PCPV), bovine papular stomatitis virus (BPSV) of cattle and parapoxvirus of red deer in New Zealand (PVNZ). Of these, ORFV, PCPV and BPSV are considered to be zoonotic since they are associated with occupational infections in humans (12, 24).

Orf infection involves mostly local skin lesions and rarely systemic infection with lesions confined mostly to the epidermis progressing through stages of erythema, papule, vesicle, pustule with scabs characteristic of the family *Poxviridae* (12). Clinically, the disease is characterised by proliferative and often self-

limiting lesions on the skin of lips, oral mucosa and around the nostrils of sheep and goats. Lesions can also sometimes be found on the teats of nursing animals and rarely on the internal organs, such as the tongue and gums of affected animals (11, 12). Furthermore, the disease occurs normally in a mild form in sheep and goats with high morbidity and significant productivity losses. Mortality ranges from 10% to 90% in lambs and kids (17) and mortality may be increased due to secondary bacterial infections (12) which warrant the development of rapid and specific diagnostics and vaccines for control of the disease to curb the economic losses.

Despite the fact that the disease is diagnosed by the characteristic clinical picture which is suggestive of the infection, it is confirmed as orf infection by a battery of serological and molecular techniques, namely: the conventional polymerase chain reaction (PCR) (15), the real-time PCR (7, 18) and genomic restriction enzyme analysis (8). Earlier, several ORFV isolates from sheep, goats, musk ox and ruminant species were characterised by phylogenetic analysis using *B2L*, an envelope protein gene, to determine the genetic similarities with different countries (1, 9, 10, 19, 25). Orf has been reported regularly with high morbidity in sheep and goats in the recent past and is considered to be enzootic in India (2, 11, 12, 13, 17). In addition, the disease has been reported repeatedly at Mukteswar in both sheep and goat flocks between 2000 and 2006 (11, 17).

The present study investigates an outbreak of orf in a small flock of sheep in Mukteswar, Uttarakhand (India), providing the clinical picture, isolation and identification of the causative agent by semi-nested *B2L* gene-based diagnostic PCR as well as by sequence and phylogenetic analyses of the *B2L* gene of isolates to identify the origin and genetic relatedness of the virus.

Materials and methods

Clinical picture of the outbreak

The outbreak occurred in a small sheep flock of 33 sheep in January and February 2009 at

Mukteswar, Uttarakhand. These animals were purchased at a local market and had no history of vaccination against orf. After 15 days of quarantine, fifteen ($n = 15$) sheep of different age groups ranging from 6 months to 2 years were affected. Clinically, the presence of skin lesions, characterised by a dermal outgrowth over the lips and mouth were observed and a nodular type was seen around oral commissures. In addition, pyrexia with inappetence were recorded. Out of a total of 15 lambs, two from affected flocks died within 8 days of the onset of infection. Serum samples were collected from all the animals whereas skin scraping and scabs were collected only from affected animals and lung lesions were sampled from dead lambs for laboratory investigation. Initially, counter-immunoelectrophoresis (CIE) was used to screen the scab materials ($n = 15$) and sera ($n = 33$) for the presence of ORFV-specific antigen and antibody, respectively, as described earlier (14). All serum samples were screened further using the serum neutralisation test (SNT) to evaluate the status of antibody titre. Positive scab samples were processed as a 10% suspension using sterile phosphate buffered saline (0.1 M) and used for DNA extraction after repeated freezing and thawing and semi-nested PCR as reported by Inoshima *et al.* (15) to confirm the aetiology of the outbreak. In semi-nested PCRs, an initial reaction was performed using PPP1 and PPP4 primers followed by PCR with PPP3 and PPP4 primer sets that resulted in a clear amplification of the 235 bp fragment as reported earlier (15).

Virus isolation and identification

Scab suspension was used for virus isolation in primary or secondary lamb teste cells grown in Eagle's minimum essential medium (EMEM, Sigma, St Louis, Missouri) supplemented with 10% new-born calf serum (Hyclone, Logan, Utah) as described by Hosamani *et al.* (10). Cells were observed daily for the appearance of virus-induced cytopathic changes, after infection and at 80% cytopathic effect (CPE); cells were harvested for DNA extraction with the AuPrep™ DNA extraction kit (Life Technologies Pvt Ltd, New Delhi) in accordance with the protocol provided by the

manufacturer. The infected cells harvested at 80% CPE were processed for electron microscopy using standard protocol (13). The total DNA was also extracted from a scab sample from an earlier outbreak (Orf Mukteswar/2000) available in the repository of the Virology Division of the Indian Veterinary Research Institute (IVRI) in Mukteswar to compare the genetic relationship with the current outbreak. The isolated DNAs were subjected to PCR amplification of *B2L* gene sequences.

Sequence and phylogenetic analysis

Amplification of the *B2L* gene from isolated total DNAs was performed as described by Hosamani *et al.* (12). In brief, PCR was performed in a 50 μ l reaction containing 2 μ l extracted DNA, 10 pmol of each primer (12), 5 μ l of the 10 \times buffer, 10 mmol/l dNTPs (deoxynucleotide triphosphates), and 0.25–0.5 IU of Taq DNA polymerase (Invitrogen, Carlsbad, California) in a thermal cycler (Mycycler, Bio-Rad, Hercules, California) with a initial denaturation step of 94°C for 3 min followed by 29 cycles of 94°C for 1 min, 52°C for 1 min, 72°C for 1 min and final extension of 72°C for 7 min.

An aliquot of the reaction was analysed by 1.5% agarose gel electrophoresis to visualise the PCR amplicons. The amplicons were then cloned into pGEMT-Easy vector (Promega, Madison, Wisconsin) and sequenced commercially in an automated DNA sequencer (ABI PRISM 3100, Perkin-Elmer, Carlsbad, California). Comparative sequence analyses were performed using published sequences of members of parapoxviruses available in the GenBank database. The details of reference sequences used in this study are given in Table I. Sequence identity at both nucleotide (nt) and deduced amino acid (aa) levels were determined using the Clustal W method of the MegAlign program using the DNASTAR package (Lasergene 6.0, DNASTAR Inc., Madison, Wisconsin). A phylogenetic tree based on deduced aa sequence of *B2L* was also constructed using the bootstrap test of phylogeny with the neighbour-joining method provided in MEGA 4.1 software (22).

Table I
Details of members of the parapoxviruses included in phylogenetic analysis

Serial No.	Virus isolate	Species affected	Country (year of isolation)	Reference	Accession No.
1	ORFV Muk/09	Sheep	India (2009)	Venkatesan <i>et al.</i> (this report)	GU139356
2	ORFV Muk 59/05	Goat	India (2005)	Hosamani <i>et al.</i> (11)	DQ263304
3	ORFV 67/04	Sheep	India (2004)	Hosamani <i>et al.</i> (11)	DQ263305
4	ORFV 79/04	Sheep	India (2004)	Hosamani <i>et al.</i> (11)	DQ263306
5	ORFV 82/04	Sheep	India (2004)	Hosamani <i>et al.</i> (11)	DQ263303
6	ORFV Hoping	Goat	Taiwan (NA)	Chan <i>et al.</i> (5)	EU935106
7	ORFV Taiping	Goat	Taiwan (NA)	Chan <i>et al.</i> (5)	EU327506
8	ORFV Nantou	Goat	Taiwan (NA)	Chan <i>et al.</i> (4)	DQ904351
9	ORFV Korea/09	Goat	Rep. of Korea (NA)	Oem <i>et al.</i> (19)	GQ328006
10	ORFV Ca USA	Goat	United States (NA)	Guo <i>et al.</i> (9)	AY278208
11	ORFV Ta USA	Goat	United States (NA)	Guo <i>et al.</i> (10)	AY424971
12	ORFV Va USA	Goat	United States (NA)	Guo <i>et al.</i> (10)	AY278209
13	ORFV Sh USA	Sheep	United States (2001)	Guo <i>et al.</i> (10)	AY424970
14	ORFV NZ2	Sheep	New Zealand (NA)	Sullivan <i>et al.</i> (21)	U06671
15	ORFV IA82	Sheep	United States (1982)	Delhon <i>et al.</i> (6)	AY386263
16	ORFV SA00	Goat	United States (NA)	Delhon <i>et al.</i> (6)	AY386264
17	ORFV Musk ox	Musk ox	United States (NA)	Guo <i>et al.</i> (10)	AY424969
18	PCPV TQ	Cow	United States (1983)	Guo <i>et al.</i> (10)	AY424972
19	BPSV RS	Calf	United States (NA)	Guo <i>et al.</i> (10)	AY424973
20	BPSV BV-AR2	Calf	United States (2000)	Delhon <i>et al.</i> (6)	AY386265

ORFV orf virus
NA not available
PCPV pseudocowpox virus
BPSV bovine papular stomatitis virus

Results

The outbreak was characterised by high rates of morbidity (45%), low mortality (6%) and the development of dermal lesions on lips and oral commissures following a phase of pyrexia and inappetence (Fig. 1). Affected animals were recovered after 4-5 weeks post infection except for two lambs that died within 10 days of showing the skin lesions. A total of 12 scabs (out of 15) and 10 sera (out of 33) from suspected clinical samples were found positive by CIE. Furthermore, when the serum samples were tested with the SNT, they showed antibody titres ranging from 1:8 to 1:32, thereby indicating the presence of orf infection. Semi-nested PCR revealed specific amplification of 235 bp product from fourteen ($n = 14$) scab samples (Fig. 2); this confirmed the aetiology of the outbreak as ORFV.

Pooled and processed scab samples produced a characteristic cytopathic effect (CPE) of ORFV in primary/secondary lamb testes cells at passage level 4 (P4), such as cell rounding, ballooning and increased refractivity and degeneration of cells (Fig. 3). The ORFV particles observed under electron microscopy are included in Figure 4. The total DNA extracted from infected cell culture fluid harvested at 80% CPE amplified 235 bp specific products in semi-nested PCR (Fig. 2) and the product was cloned and subsequently sequenced confirming the fidelity of the amplicon (Accession No. GU139359).

Further, PCR amplification of the *B2L* gene from scab materials collected from two outbreaks showed specific products of 1206 bp in size with an open-reading frame (ORF) of 1 137 bp in length (Fig. 5). Edited sequences of the virus isolates (ORFV Muk TS/09; ORFV Muk/2000) were submitted to National Center



Figure 1

Clinical picture of orf virus infection in sheep

- A. Affected sheep showing multiple nodular lesions on the upper and lower labia and nose
- B. and C. Proliferative growth on the oral commissure
- D. Nasal, oral and ocular mucous discharge
- E. and F. Lesions on inside of lips and tongue

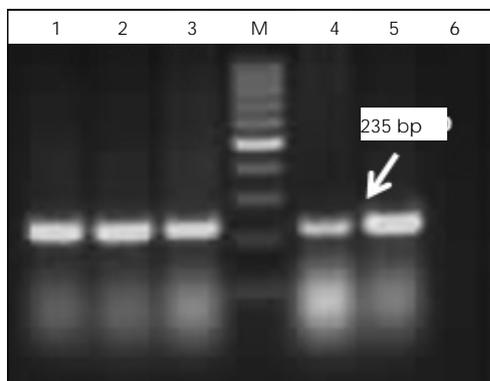


Figure 2

Agarose gel electrophoresis of semi-nested polymerase chain reaction products of orf virus DNA

- Lane 1: positive control (Orf Mukteswar 59/05) showing amplification of 235 bp product from viral DNA
- Lanes 2, 3, 4 and 5: clinical samples
- Lane 6: negative control showing lack of amplification in the reaction that contained no template DNA
- Lane M: 100 bp DNA ladder (MBI Fermentas, Maryland)

for Biotechnology Information (NCBI) GenBank (Accession Nos GU139356 and HM466933). Sequence analysis of the *B2L* coding gene sequence shares a 98.1-99.8 and 98.4-99.7% identity with Indian ORFV isolates with a maximum identity of 99.8-99.7% to goat isolate (ORFV Muk/2000) at nt and aa levels, respectively. It also shares 98.3 and 98.2% identity with Indian vaccine strain (ORFV Muk 59/05), respectively at nt and aa levels. Besides, a percent identity of 84.9-85 and 83.4 with BPSV and 95.3 and 95.5 with PCPV was observed, at nt and aa levels, respectively. The overall percentage of nt and aa identity among ORFV isolates and other parapoxviruses are presented in Table II. A phylogenetic analysis also revealed a cluster of ORFV isolates that was distinct from other members of the genus (Fig. 6) and when compared to Indian ORFV isolates, it was closest to goat isolate (ORFV Muk/2000) as mentioned above.

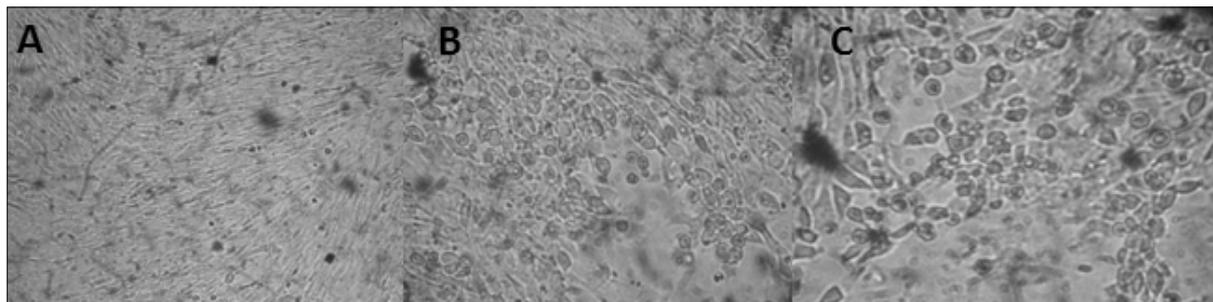


Figure 3
Micrograph of characteristic cytopathic changes of the virus isolate (orf virus Muk/09, passage 4) in secondary lamb testis cultures on day 4 post-infection
A. Healthy mock infected lamb testes cells
B. Characteristic rounding and ballooning of cells
C. Increased refractivity of cells accompanied by degenerative changes

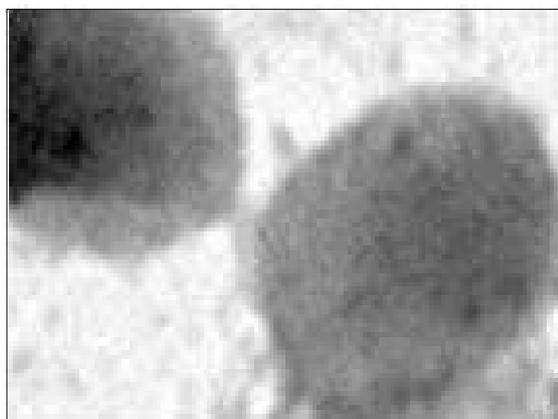


Figure 4
Electron micrograph showing typical cocoon-shaped orf virus particles isolated from clinical samples

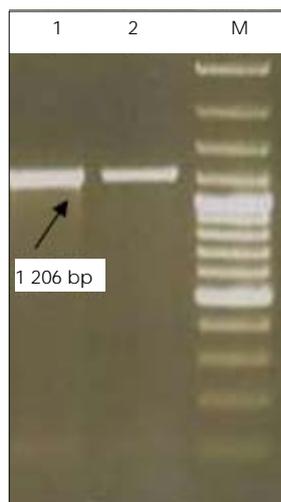


Figure 5
Agarose gel electrophoresis showing amplification of *B2L* gene
Lane 1: Viral DNA isolated from orf virus Muk TS/09
Lane 2: Orf virus Muk/2000 and 100 bp DNA ladder
M: marker (MBI, Fermentas, Maryland)

Discussion

Orf is one of the economically important pox diseases of sheep and goats and is enzootic in India. In our study, the outbreak inflicted moderate morbidity (45%) and low mortality (6%) in the affected flock (11, 17). However, it can be severe in lambs and kids with mortality reaching 80%-90%, depending on the host, environment and pathogen factors and their interactions (12). The clinical picture of the outbreak was clearly indicative of orf infection (11, 13).

Initially, clinical samples, such as skin scabs and sera, were screened for parapoxvirus-specific antigen and antibody, respectively using CIE and the SNT (14). The SNT titres ranged from 1:8 to 1:32 in 14 sera samples tested, thereby indicating orf infection and seroconversion (12). The pooled tissue samples which were positive to CIE and *B2L* gene-based semi-nested PCR, produced a characteristic CPE in cell culture as reported by Hosamani *et al.* (11, 12). A specific amplification of 235 bp PCR amplicons (Fig. 2) in diagnostic PCR revealed the specificity of the isolate (11, 12, 13).

Diagnosis of orf infection in sheep and goats has generally been performed based on characteristic skin lesions and also by electron microscopy (17) but, the semi-nested PCR based on the envelope protein gene (15) and loop-mediated isothermal amplification (23) have proved to be more rapid and sensitive for the detection of ORFV DNA from clinical

Table II
 Percent identities of *B2L* coding sequences of orf virus Muk TS/09 isolate with other members of the genus *Papapoxvirus*

Virus	<i>B2L</i> gene-orf	
	Nucleotide	Amino acid
Among Indian orf virus isolates	98.1-99.8	98.2-99.7
Among isolates from Mukteswar (2000-2006)	98.3-99.8	98.2-99.7
With vaccine strain (orf virus Muk 59/05)	98.3	98.2
With orf virus Muk/2000 isolate	99.8	99.7
Among Indian isolates of sheep origin	98.9-99.1	98.4-99.2
Among Indian isolates of goat origin	98.1-99.8	98.2-99.7
Among Asian orf virus isolates	97.7-98.1	96.6-97.9
Among other orf virus isolates worldwide	97.6-98.7	96.6-98.2
Orf virus from Musk ox	98.0	97.1
Pseudocowpox virus	95.2	95.5
Bovine papular stomatitis virus	84.9-85.0	83.4

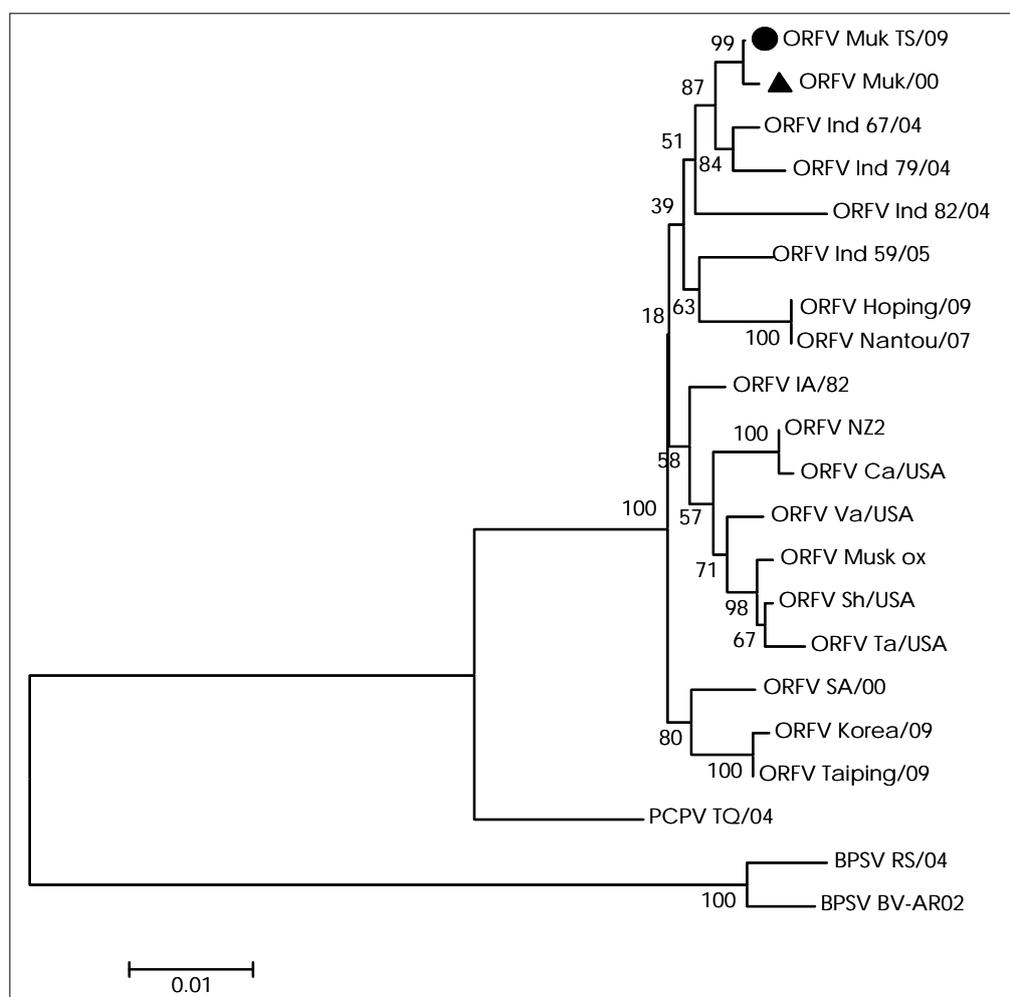


Figure 6
 Phylogenetic analysis of different parapoxviruses based on *B2L* amino acid sequence
 Unrooted tree was constructed using neighbour-joining method of bootstrap test of phylogeny in the MEGA version 4.1 program
 Numbers on the tree branches represent the bootstrap support calculated per 1 000 bootstrap replicates
 The scale bar beneath the tree represents the amino acid substitutions per site

samples, in addition to the *A32L* gene-based single-step PCR for the differentiation of genotypes of orf viruses (5).

Many structural and non-structural genes of parapoxvirus isolates of diverse host species have been characterised and phylogenetic analyses have revealed close genetic relationships with members of the genus *Parapoxvirus* (1, 4, 9, 10, 11, 13, 16, 25). Among them, *B2L* is a highly immunogenic envelope protein encoded in the genome of the genus *Parapoxvirus*, homolog of vaccinia virus envelope protein antigen-P37k (21). Therefore, in the present study, *B2L* gene sequences of both isolates (ORFV Muk TS/09 and ORFV Muk/2000) were compared and analysed with other ORFV and members of the genus *Parapoxvirus*. They shared a maximum percent identity of 99.8 and 99.7 among Indian ORFV isolates whereas they revealed a 97.6-98.7 and 96.6-98.2% identity from ORFV isolates reported worldwide at nt and aa levels, respectively. Unique aa changes observed in the coding region of *B2L* protein are I245V (only in ORFV Muk/2000, ORFV Muk TS/09 and ORFV 79/04 isolates) and D294N (all Indian isolates except ORFV Muk 59/05) when compared with other ORFV isolates and other members of the genus *Parapoxvirus*.

Earlier, there were three orf outbreaks at Mukteswar, namely: Muk/2000 (goats), Muk 59/05 (goats) and Muk 67/04 (sheep) that were reported in sheep and goat flocks with significant mortality and morbidity rates. The major source of infection suspected was the transportation of animals (11, 17).

Sequence analyses based on the *B2L* gene were used to determine the origin and genetic relatedness of these isolates from the same geographic niche from outbreaks that occurred between 2000 and 2009. These analyses showed a percent identity of 98.3-99.8 and 98.2-99.7 at nt and aa levels with these isolates, respectively, and also revealed that the current isolate of sheep origin (ORFV Muk TS/09) was very closely related to goat isolate (ORFV Muk/2000). Moreover, all the isolates of ORFV from sheep and goats of Indian origin are closely related and form a single group in the phylogenetic tree.

Phylogenetic analysis showed that the currently isolated ORFV clustered with other Indian isolates and is closely positioned with ORFV Muk/2000 and is distinct from other members of the genus (Fig. 5) as reported by Hosamani *et al.* (11). This analysis is a valuable tool for the identification and differentiation of parapoxviruses that are closely related antigenically (12).

Conclusions

In this investigation, various diagnostic assays identified the aetiology of suspected cases of orf infection in small sheep flocks as contagious ecthyma. Furthermore, sequence and phylogenetic analyses of the *B2L* gene revealed the genetic relationship with other Indian isolates. When compared to three isolates from same geographic region, including vaccine strains, analyses revealed a close genetic relationship to a goat isolate from an outbreak which occurred in Mukteswar 2000. Consequently, the Mukteswar isolates need to be investigated thoroughly using other genes that are involved in the structural or functional nature of proteins, especially the antigenic or immunogenic aspects, to identify these frequent outbreaks in sheep and goats in the same geographic area. Besides, several outbreaks of orf (scabby mouth), either alone or in combination with other opportunistic pathogens, have been reported frequently in India and have often been misdiagnosed or have passed unnoticed due to the presence of other infections (12).

Given the importance of orf infection and on account of the productivity losses inflicted on sheep and goat populations, outbreaks need to be monitored carefully. This necessitates systematic investigation of infections, identification of aetiology using specific diagnostic PCR, preferably of multiplex format, and targeting the other viral infections and vaccination of susceptible populations using recently developed IVRI live-attenuated orf vaccine (3) either single or combined with PPR and/or goat pox vaccines (3). This will limit the spread and severity of outbreaks and thus will reduce the economic impact of disease.

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