Isolation and molecular characterization of *Orf virus* from natural outbreaks in goats of Assam

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Abstract Outbreaks of contagious ecthyma (caused by a *Parapox virus*) in goats were investigated in 6 districts of Assam, a north eastern state of India. Diagnosis of the disease was carried out employing both standard virological as well as molecular methods. Four representative isolates from different places were selected for phylogenetic analysis. The major envelop protein (B2L) of *Orf virus* was targeted for molecular analysis. The sequencing and phylogenetic analysis of the selected sequences at nucleotide level revealed that the *Orf virus* isolates were closely related to each other (97.6–100 %) and showed highest similarity to the *Orf virus* isolate 82/04 (98.4 %), reported from Shahjahanpur, India. The data will provide an insight in transmission of the virus from northern to North eastern part of the country.

Keywords Contagious ecthyma · Goat · *Orf virus* · B2L gene · Phylogenetic analysis · Assam

Contagious ecthyma (CE) or most commonly orf is a contagious non-systemic eruptive viral skin disease that occurs worldwide in small ruminants affecting mostly sheep and goat [1]. The etiological agent, *Orf virus*

(ORFV) is an epitheliotropic double-stranded DNA virus of genus *Parapoxvirus* (PPV), which represents one of the ninth genera within the *Chordopoxvirinae* subfamily and *Poxviridae* family. Parapoxviruses are morphologically distinguished from other members of poxvirus genera by their ovoid shape with a single thread like surface tubule surrounding the particle in a crisscross pattern and relatively small size [2]. This genus 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) [3]. The ORFV genome consists of linear double-stranded DNA about 138 kb long with ~64 % G+C content, containing 132 putative genes that included 89 highly conserved genes and some variable genes [4, 5]. The enveloped gene B2L (ORF 011) of the virus encodes a highly immunogenic major envelope protein of 42 kDa which has been extensively studied for molecular characterization and phylogenetic analysis of strains of the virus [6]. *Orf virus* encodes range of molecules that play vital role in immune evasion by way of production of anti-inflammatory proteins such as virus interferon resistance protein (OVIFNR), GM-colony stimulating factor (GM-CSF) and IL-2-inhibitory factor (GIF), virus interleukin 10 orthologue protein (vIL-10) and vascular endothelial growth factor (VEGF) [7].

The clinical disease is characterized by proliferative lesions that commence on the lips, muzzles, muco-cutaneous junctions, nostrils, gums and sometimes spread to internal organs including tongue. Dermal lesions may involve other parts of the skin, including the face, eyelids, ears, under the tail, coronets, udders of nursing ewes or on the genitalia [8]. The disease usually lasts for 3–4 weeks and resolves spontaneously in 1–2 months. Although the mortality rate in uncomplicated cases rarely exceeds 1 %, but morbidity of the disease is very high approaching up to

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100 % [9]. However, secondary complications and mixed infections with other viruses and bacteria can increase the mortality rate from 20 to 50 % [6]. The disease has a significant economic impact on rural communities where the marginal farmers are predominantly reliant upon live-stock farming as a source of livelihood.

Contagious ecthyma is endemic in most parts of Assam, but is only occasionally reported. The farmers were interviewed through a questionnaire designed for collection of information regarding pox viral diseases and were found that they were totally unaware about pox viral diseases in animals and as such no systematic control policies are being followed so far. There is little information on the characterization of ORFV strains that are endemic in Assam. Further no prospective epidemiological picture has been elucidated.

In this study outbreaks of contagious ecthyma, their detection, isolation and molecular epidemiological investigation based on the B2L gene of ORFV isolates originated from goat flocks at different districts of Assam, a northeastern part of India were investigated.

An investigation of CE outbreaks was conducted from September 2013 to July 2014. Materials were taken from different outbreaks of orf that occurred in goats of 6 districts of Assam. Lesions were characterized by proliferative extensive dermal outgrowths covering the muzzle of the affected goats which were mostly confined to the skin around the mouth, the gums and the inner surface of the lips (Fig. 1a, b, c). Clinical examination of the these animals revealed elevated temperatures ranging from 40 to 41 °C. The affected number of animals were grouped into 0–2, 2–4, 4–6 and above 8 months. A total of 52 scab samples were collected from 11 natural outbreaks from different parts of Assam.

Suspected clinical samples, skin scabs ($n = 52$) from the affected animals were collected for confirmatory diagnosis of orf (Table 1). The processed scab materials were used for DNA Extraction employing DNeasy Blood and Tissue Kit (Qiagen) following manufacturer's protocol.

Scab samples were initially screened for presence of ORFV-specific antigens by means of countercurrent immunoelectrophoresis (CIE) [10] and Semi-nested PCR [11] using primer sets PPP-1(5'-GTC GTC CAC GAT GAG CAG CT-3'), PPP-3(5'-GCG AGT CCG AGA AGA ATA CG-3') and PPP-4 (5'-TAC GTG GGA AGC GCC TCG CT-3'). Sn-PCR was performed with few modifications in annealing of primers with an initial denaturation step 95 °C for 9 min followed by 29 cycles of 94 °C for 1 min, 52 °C for 1 min and 72 °C for 1 min.

Scab samples collected from affected animals were ground in sterile pestle and mortar in approximately 1.5 ml of phosphate buffered saline (0.1 M, pH 7.4) and frozen and thawed thrice as per standard method [12]. The supernatant fluid was collected after centrifugation at 1500 rpm for 15 min (Spinwin, Tarsons). Prior to inoculation, the supernatant was treated with antibiotic–antimycotic solution (Sigma Aldrich) at the rate of 100 units per ml of supernatant fluid. Primary Lamb Testes (PLT) cells at Passage-15 was obtained from Pox virus laboratory, IVRI, Mukteswar. The cells were maintained in the laboratory by continuous subculturing using Dulbecco's Modified Eagle's Medium (DMEM; Sigma Aldrich) containing 5 % Fetal Calf Serum (Hyclone). The cell monolayer was split into 1:2 ratio, which yielded confluent growth of 80–90 % within 24 h of sub-culturing. Clinical samples ($n = 4$) found positive in both CIE and Sn-PCR were randomly selected for isolation of viruses in Primary Lamb Testes (PLT) cells. Each virus was propagated through blind passages until *Orf virus* specific cytopathic effects were observed. Propagation of the viruses in PLT cells were confirmed by *Orf virus* specific CPE in Hematoxylin-Eosin (H&E) and May Grunwald Giemsa (MGG) staining and Conventional PCR targeting the full length B2L gene of *Orf virus*.

Full-length B2L gene was amplified by conventional PCR from the DNA extracted from cell culture harvest of ORFV isolates using previously published primer sets: OVB2LF1, TCCCTGAAGCCCTATTATTTTGTG and

Fig. 1 Clinical picture of orf in infected goats. **a** Proliferative lesions extending into the gums of affected goat. **b** Moderate lesions on the oral commissures. **c** Affected goat showing nodular lesions on the lips and nose

Table 1 Details of clinical samples collected from the outbreaks

Outbreak no.	Place of outbreak	Number of samples collected	CIE (%)	Sn-PCR (%)	Isolation
1	Laluk, Lakhimpur,	5	2	5	+
2	Byrnihat, Kamrup	9	1	8	+
3	Khanapara, Kamrup	4	–	–	NA
4	Byrnihat, Kamrup	6	1	6	NA
5	Demow, Sivasagar	3	1	3	+
6	Karbi Anglong	2	–	–	NA
7	Hajo, Kamrup	6	1	3	+
8	Dhemaji	3	–	3	NA
9	Azad, Lakhimpur	3	1	3	NA
10	Laluk, Lakhimpur	9	–	9	NA
11	Nalbari	2	–	–	–
Total	11 outbreaks	52	7 (13.46 %)	40 (76.92 %)	

CIE Counterimmunoelectrophoresis, Sn-PCR semi-nested polymerase chain reaction, NA not applicable, + positive, % percent positive

OVb 2LR1, GCTTGCGGGCGTT CGGACCTTC [13]. Amplification of B2L gene from isolated DNA was performed in standard 25 µl reaction containing 3 µl extracted DNA, 20 pmol of each primer, 2.5 µl of 10× PCR buffer, 25 mM of MgCl₂, 10 mM deoxynucleotide triphosphates (dNTPs) and 2.5 IU of DyNzyme (Thermo Scientific) in a thermal cycle (Perkin Elmer, Applied Biosystems) with an initial denaturation step of 94 °C for 5 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. PCR amplicons were purified using QIA quick Purification kit (QIAGEN) as per manufacturer's instructions. The amplicons were cloned into pGEMT-Easy vector (Promega, Madison, WI, USA) and transformed into High Efficiency JM109 competent cells provided with the kit. Clones thus generated were sequenced commercially at DNA sequencing services, Merck Technologies Limited, Bangalore. Comparison of the B2L gene sequences of Assam isolates was carried out with published sequences of members of PPVs available in the GenBank database. Sequence identity at nucleotide (nt) level were determined by Clustal W method of Meg-Align program in DNASTAR package (Lasergene 6.0, DNASTAR Inc., USA). The phylogenetic and molecular evolutionary analyses were conducted using program MEGA 5.04. The details of the PPV isolates included in the analysis are listed in Table 2.

Outbreaks of orf were recorded from 11 different places of Assam which more or less occurred at the same time, highest being three outbreaks in Lakhimpur district of upper Assam. All the age groups of goat were affected with the classical form, a few among them were affected by an aggravated form of the disease where proliferative dermal outgrowths extended into the gums of the affected goats

(Fig. 1a). Both moderate and severe lesions were observed in all the age groups of the affected animals (Fig. 1b, c). In all the outbreaks it was observed that maximum number of affected animals were under the age group 8 months of age. The affected animals from the outbreaks recovered after 2–3 weeks post infection without any mortality. However, no seasonal variation on occurrence of the disease among the goat population of Assam was observed as the disease was prevalent throughout the year.

The presence of ORFV in clinical samples was initially screened by countercurrent immunoelectrophoresis (CIE) where 7 (13.46 %) samples were found positive. Further, to confirm the etiology of the outbreaks as ORFV, Sn-PCR was performed using primers PPP-1 and PPP-4 which resulted an amplification of 594 bp followed by PCR with PPP-3 and PPP-4 primer sets that resulted in clear amplification of 235 bp of B2L gene of ORFV. A total of 40 (76.92 %) out of 52 scab samples were found positive in Sn-PCR.

A total of 4 (four) clinical samples found positive in both CIE and Sn-PCR were processed for isolation of *Orf virus*. Samples treated with antibiotic–antimycotic solution when incubated for an hour, completely eliminated bacteria and fungi present in the suspension. The virus recovered from the scab samples was used to infect primary lamb testes cells but no cytopathic effect (CPE) was observed even up to three blind passages. Virus induced cytopathic changes were observed from the fourth passage and was initiated by day 2 post inoculation (dpi) and progressed to 80 % by day 4–5. CPE was characterized by cell rounding, ballooning, granulation and vacuolation of the cytoplasm within 48 h post inoculation (hpi) in virus-infected cells but not in mock-infected cells. Cell degeneration and

Table 2 Information about *Parapoxvirus* strains (n = 26) used for phylogenetic analysis

Strain identification	Collection year	Species affected	Accession no.	Country of isolation
Orf Assam By26/13	2013	Goat	This report	India
Orf Assam NLP_35/13	2013	Goat	This report	India
Orf Assam Hajo_21/13	2013	Goat	This report	India
Orf Assam SBR_16/13	2013	Goat	This report	India
ORFV-Assam/10	2010	Goat	JQ040300	India
ORFV-Shanhjahnpur 82/04	2004	Goat	DQ263303	India
ORFV-Muk/2000	2000	Goat	HM466933	India
ORFV-Mukteswar 67/04	2004	Sheep	DQ263305	India
ORFV-Izatnagar 79/04	2004	Sheep	DQ263306	India
ORFV-Cam/09	2008	Camel	GU460370	India
ORFV-ovine isolate	NA	Sheep	AY424970	USA
ORFV-Turkey (ORF3/2007)	2007	Goat	KC491191	Turkey
ORFV-Nantou	NA	Goat	DQ904351	Taiwan
ORFV-Taiping	NA	Goat	EU327506	Taiwan
ORFV-Hoping	2008	Goat	EU935106	Taiwan
ORFV-NZ2	1993	Goat	JN088051	Brazil
ORFV-D Brazil	1992	Sheep	JN088052	Brazil
ORFV-LiaoNing	2010	Goat	HQ694773	China
ORFV-Shanxi	2009	Goat	HQ202153	China
ORFV-JS04	2006	Sheep	GU903501	China
ORFV-Jilin	2008	Sheep	FJ808074	China
ORFV-Korea/2009	2009	Goat	GQ328006	Korea
ORFV- F92.849R	NA	Reindeer	JF773697	Finland
PCPV- SV723-12	2012	Cattle	KC896642	Brazil
BPSV-Reference strain	NA	Calf	AY424973	Not available
BPSV-2/07	2007	Calf	KF478806	Germany

detachment of the monolayer was observed by day 6–7 post dpi. The cell culture supernatant from the fourth passage was further used to reinfect fresh primary lamb testes cells where CPE was observed within 48 hpi, similar to the CPE observed in the fourth passage.

In order to further confirm the isolation of viruses, the full length B2L gene of ORFV was amplified which resulted specific product of 1206 bp (open reading frame of 1137 bp). The PCR product was subsequently purified using QIA quick Purification kit (Qiagen, Hilden, Germany) and cloned into pGEM-T vector (Promega, Madison, WI, USA) using TA cloning mechanism. Successful cloning of the inserts resulted into recombinant clones, which was identified by colour screening on indicator plates. The transformed recombinant colonies were screened by conventional PCR and out of six white colonies, two colonies were found positive resulting a clear amplification of major enveloped protein gene (1206 bp). Both forward and reverse PCR primers were used to directly sequence the cloned PCR products (Merck, Bangaluru, India).

To compare and determine the phylogenetic relationship of ORFV strains of Assam with other ORFV strains, each of the sequences of B2L gene of isolated Orf viruses (Orf

Assam By 26_13, Orf Assam NLP_35/13, Orf Assam Hajo_21/13 and Orf Assam SBR_16/13) were subjected to NCBI BLAST search. Corresponding sequences (n = 22) that showed high sequence identities were retrieved from NCBI database as shown in Table 2. Sequence identity at nucleotide level was determined using Clustal W method of MegAlign software. Using nucleotide sequences of entire B2L gene in the GenBank, phylogenetic relationship between the Assam isolates and other isolates were constructed by neighbor-joining method and bootstrap analysis (Fig. 2). On sequence analysis, it was revealed that Orf virus isolates from goats of Assam (Orf Assam By_26/13, Orf Assam NLP_35/13, Orf Assam Hajo_21/13 and Orf Assam SBR_16/13) showed a high degree (97.6–100 %) of sequence identity among themselves. All four isolates shared maximum identity (97.5–100 %) with the previously reported *Orf virus* isolate from Assam (JQ040300_Assam). From the analysis, it was evident that a high degree of sequence similarity (95.0–98.4 %) exists between the *Orf virus* isolates of the present study with that of the *Orf virus* isolates reported from India. Isolates of the present study showed 95.0–98.4 % and 95.1–98.3 % similarities with Indian isolates of goat and sheep origin respectively. Among the Indian

Fig. 2 Phylogenetic analysis of different *Parapoxviruses* based on deduced nucleotide sequences of full length B2L gene. The phylogenetic relationship of nt sequences was constructed by using the neighbor-joining program of MEGA version 5.04. The isolates Orf Assam By26_13, Orf Assam Hajo21_13 Orf Assam NLP35_13 Orf Assam SBR16_13 are closely related and lies within the same group of previously reported isolate of Assam (JQ040300_Assam) indicated with pink circles and blue diamonds (also for other Indian ORFV), respectively

Orf virus isolates, they showed maximum identity (98.4 %) with that of Indian ORFV isolate 82/04 (DQ263303_Shahjahanpur). Further, they shared an identity of 91.0–94.2 %, 93.2 % and 82.4–84.4 % at nucleotide level with Orf isolate of camel origin, PCPV and BPSV respectively.

Contagious ecthyma is endemic in Assam and no vaccination program is performed to control the disease. In recent years there have been increasing reports of ORFV infections. The classical clinical disease is manifested as scabby lesions around the lips, mouth, muzzle, nostrils, teats, and oral mucosa. The lesions start initially as erythematous spots or swelling, followed by formation of papules, which become bigger and pustular with a yellowish creamy appearance. These papules progress to develop into scabs [14]. In this outbreak, the lesions were confined to the mouth, the gums and the inner surface of the lips (Fig. 1a, b, c). These classical clinical signs are strong evidence of *Orf virus* infections. Among the different districts of Assam,

distribution of orf was found to be highest in Lakhimpur district where outbreaks in goats were more severe in comparison to other places under study. Lakhimpur district shares inter-state boundary with Arunachal Pradesh and borders with its nearby districts Dhemaji and Sonitpur. Unrestricted animal movements across the state boundaries in search of pasture lands and more number of goat population along with improper husbandry practices may be the key factor for more severe outbreaks at Lakhimpur. In all the attended cases, morbidity was found to be high (95–100 %) and mortality was low (10.76 %) which is greatly affecting the economic status of the farmer due to poor growth as well as death of young ones.

Diagnosis of ORFV antigens in goats are characteristically based on clinical symptoms and electron microscopy, but semi-nested PCR based on major envelope protein and isothermal amplification have proved to be more rapid and sensitive for detection of ORFV from clinical samples [15].

In our investigation, Semi-nested diagnostic PCR was performed to detect ORFV infections in 52 scab samples, out of which 40 samples were found positive. Specific amplification of 594 bp with primer sets PPP-1 and PPP-4 in first PCR and subsequent amplification of 235 bp with primer sets PPP-3 and PPP-4 revealed the specificity of ORFV infection. The etiological agent was further characterized by isolation in cell culture and nucleotide sequence studies. Isolation of ORFV in cell culture is regarded as the “gold standard” method for detection of *Parapoxviruses*. Among the primary cell cultures, primary lamb testes and primary lamb kidney are the most common [8]. However, other cell lines such as Madin-Darby ovine kidney cells [16], Ovine fetal turbinate cells [3] and Vero Cells [17] have also been used for ORFV isolation. Virus induced cytopathic effects (CPE) were cell rounding, ballooning, increased refractivity and degeneration of cells. Studies by previous workers observed CPE after 3–4 blind passages [15]. In our investigation, the samples which were selected for isolation of *Orf virus* produce similar CPE with that of the previous reports by day 2 post inoculation, characterized by rounding, ballooning, vacuolation of the cytoplasm, degeneration and finally detachment of the monolayer by day 5–6. Virus isolation was confirmed by conventional PCR targeting the full length major enveloped protein gene, which resulted an amplification of B2L gene with an expected DNA fragment of 1206 bp of *Orf virus*. These results matched the results of orf viruses reported earlier from different parts of India and throughout the world [2, 3, 6, 15].

ORFV is environmentally stable, highly contagious, and transboundary pathogen. The intrinsic immune evasion properties of ORFV coupled with inefficient control measures had probably contributed to the increasing number of outbreaks reported both in sheep and goats [18]. A limited attempt to characterize these isolates had resulted in poor understanding of the molecular epidemiology of the orf outbreaks particularly from Assam [6]. In the ORFV genome, ORFV011, 059, 109, 110 and 132 genes have distinct functions for *Orf virus* infections and pathogenesis. The ORFV011 gene encodes the major envelope immunogenic protein (B2L). Sequence analyses based on the ORFV B2L gene were widely used to determine the origin and genetic relatedness of these isolates from the same geographic niche [19]. Therefore, in our present study, orf viruses were isolated from field outbreaks and the B2L gene of the isolates were targeted for molecular characterization of the isolates. A total of four ORFV isolates, viz., Orf Assam By_26/13, Orf Assam NLP_35/13, Orf Assam Hajo_21/13 and Orf Assam SBR_16/13 were selected randomly for molecular characterization by cloning and sequencing of B2L gene. On sequence analysis, it was revealed that all four isolates shared 97.6–100 % identity among them at

nucleotide level. The isolates Orf Assam By 26/13 and Orf Assam NLP 35/13 while other two Orf Assam SBR 16/13 and Orf Assam Hajo 21/13 showed 100 % identity with each other. All four isolates are closely related to previously reported *Orf virus* isolate from Assam [6], supporting the clue of circulation of same virus among the goats of Assam. Comparative sequence analysis of the B2L gene of all four isolates of Assam with other ORFVs reported from rest of India as well as from other parts of world, also revealed close relationship among them as indicated by their similarity percentages at nucleotide level. Among the Indian *Orf virus* isolates, they showed maximum identity (98.4 %) with that of Indian ORFV isolate 82/04 (DQ263303_Shahjahanpur). In the phylogenetic analysis also, all four ORFV isolates from Assam were clustered along with the other ORFVs reported worldwide (Fig. 2). This finding is also in accordance with the earlier report of phylogenetic analysis of ORFV isolate from Assam [6]. The reasons for circulation of ORFV into adjacent areas were mainly marketing of flocks from nearby areas, migration of sheep and goat flocks in search of pastures and most importantly inefficient control strategy.

Currently, there are few data available on the incidence of this disease and number of animals that receive treatment annually. The morbidity and economic loss are significant because farmers of Assam depends on livestock farming which include small ruminants like goats and sheep. In such situations, a continued epidemiological surveillance and monitoring is of great concern. Awareness campaigns regarding pox viral diseases should become a routine element of extension programmes for farmers. Moreover, more numbers of isolates and their characterization will help in understanding the epidemiology of orf in goats of Assam.

Contagious ecthyma infection in goats of Assam has been found to be very common as indicated by consequential outbreaks attended during the study period. The etiology of the disease was confirmed as *Orf virus* based on clinical signs, PCR amplification, virus isolation and nucleotide sequencing. Furthermore, phylogenetic studies revealed that ORFV isolates from Assam clusters together with previously reported isolate from Assam (JQ040300_Assam) and also with other ORFVs reported worldwide, especially the “ORFV Shahjahanpur 82/04” isolated from India. In Assam, the goat farming is getting popular among the farmers owing to its importance as meat animal in the state. However, occurrence of such diseases like contagious ecthyma is not only affecting the economic status of the farmers but also the demand for meat in the state. The data generated in the present study may provide an insight to the transmission and epidemiology of orf virus among goat population of Assam and shall help in formulating the control strategies against the disease.

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