

Isolation and characterization of Indian isolates of camel pox virus

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Abstract In this study, we isolated and identified three camel pox viruses (CMLV) from two outbreaks of camel pox infection in camels associated with eruptions on cheeks, nostrils, limbs, scrotum, and sheath that occurred at different places of Bikaner district, Rajasthan (India). The scab specimens collected were subjected for virus isolation in Vero cell culture, and the isolated viruses were characterized by employing polymerase chain reaction (PCR) and sequencing. The causative agent was identified as CMLV, based on A-type inclusion, B5R and C18L genes-specific PCRs and partial sequencing of these genes, which clearly confirmed that the outbreaks were caused by CMLV and

identity of CMLV isolates. Further, phylogenetic analysis of partial C18L gene sequences have showed that Indian CMLV are clustered together with other reported isolates/strains.

Keywords Camel pox · Virus isolation · Identification · Characterization · PCR · Sequence analysis

Abbreviations

ATI	A-type inclusion
CMLV	Camel pox virus
CPE	Cytopathic effect

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MEGA 4	Molecular evolutionary genetic analysis version 4
PCR	Polymerase chain reaction

Introduction

Camel (*Camelus dromedaries*) is an important animal of the desert ecosystem. The camel in India is mostly confined to Rajasthan, Haryana, Punjab, Gujarat, Madhya Pradesh, and Uttar Pradesh (Marodam et al. 2006). Camel pox is contagious, often sporadic, and notifiable to Office International des Epizootics. Generalized viral skin infection of camels is frequently seen in young animals aged 2 to 3 years in Africa, the Middle East, and southwest Asia. In these areas, the camels are used as beast of burden and for milk. The disease has been reported initially in Punjab and Rajaputana (India) (Leese 1909) and later from many other neighboring countries like Afghanistan, Pakistan, Saudi Arabia, other countries like UAE and Soviet Union, Iran, Egypt, Kenya, Iraq, Somalia, Yemen, Morocco, Ethiopia, Oman, Sudan, and Niger including India (Chauhan and Kaushik 1987; Marodam et al. 2006).

The outbreak in a herd is very often associated with weaning or poor nutrition, with fatal severe form occasionally. Disease occurrence is accompanied with morbidity, mortality, and case fatality rates respectively of 30–90%, 1–15%, and 25% (Al-Ziabi et al. 2007). The causative agent, camel pox virus (CMLV), was earlier thought as a zoonotic agent (Leese 1909), but so far a little evidence has been documented from Somalia in smallpox unvaccinated individuals (Kriz 1982). The incubation period varies from 4 to 15 days with an initial rise in temperature followed by papules on labia, vesicles, pustules, and finally formation of scabs. Camel pox infection is diagnosed based on clinical symptoms, isolation, and electron microscopy and differentiated from other orthopoxviruses (OPXVs) by employing either restriction enzyme analysis or C18L gene-based PCR (Balamurugan et al. 2009). Various cell lines such as Hela, GMK-AH1, BSC-1, WISH, and Vero are being used for isolation of viruses. In the present investigation, the causative agent of camel pox outbreaks, which occurred in Bikaner district, (Rajasthan) India, was isolated in Vero cell line and identified by characteristic cytopathic effects (CPE) and confirmed by A type inclusion (ATI), B5R (major outer envelope protein), and C18L (ankyrin repeat protein) genes-based diagnostic PCRs and sequence and phylogenetic analysis.

Materials and methods

The suspected camel pox clinical samples were collected from two separate outbreaks that were associated with

considerable morbidity occurred in two villages of Bikaner, Rajasthan (India) during 1997. The isolates (CMLV 1 and CMLV 2) from these two outbreaks were recovered in Vero cells at the National Biotechnology Centre, Indian Veterinary Research Institute (IVRI), Izatnagar, Bareilly, Uttar Pradesh. The third isolate, CMLV-Hyd 06 sample, was originally collected from a male camel aged 10 years from Bhutan-Ka-Bass, Bharou, Bikaner, Rajasthan, on 14th Dec, 2002. The animal exhibited symptoms of eruptions on cheeks, nostrils, limbs, scrotum, and sheath. This animal was brought to Bikaner Veterinary College Hospital, Rajasthan for treatment. Scabs from healing ulcers/eruptions were collected in 50% glycerol saline and sent to M/s Indian Immunologicals Limited (IIL), Hyderabad, Andhra Pradesh, for isolation and adaptation in Vero cells (Marodam et al. 2006). However, this isolate was not characterized genetically then.

The suspected scab/skin biopsy samples were triturated and a 10% suspension was made in Earle's minimum essential medium (pH, 7.4). After, three cycles of freezing and thawing, the homogenate was clarified at 1,500×g for 10 min and treated with penicillin (10,000 IU/ml), streptomycin (100 µg/ml), and mycostatin (50 IU/ml) before filtration through a 0.45-µm membrane filter and stored at –80°C until further use. Filtered supernatant (500 µl) was used to infect a 48-h-old Vero cell monolayer in 25-cm² flask as per standard protocol and cells were observed daily for the appearance of CPE.

Further, three rabbits each were inoculated with 0.5 ml of cell culture harvests (CMLV 1, CMLV 2, and CMLV-Hyd isolates) intradermally for studying the pathogenesis. The rabbits were observed for a period of 7 days for the appearance of clinical symptoms and lesions. Similarly, six mice, of which two were inoculated with each CMLV

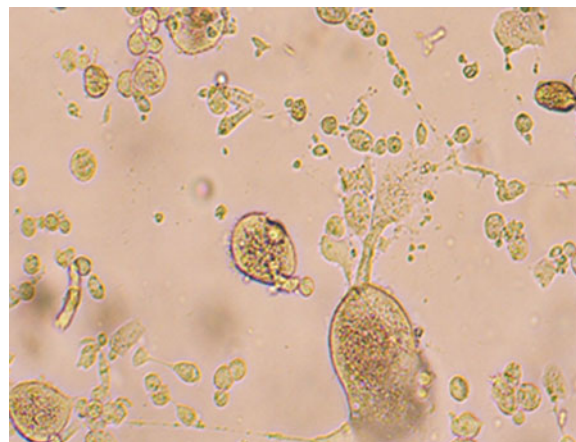


Fig. 1 CMLV 1-infected Vero cells showing characteristic cytopathic changes such as rounding, ballooning, and syncytia with degenerative changes on day 3 post-infection. ×400 magnifications

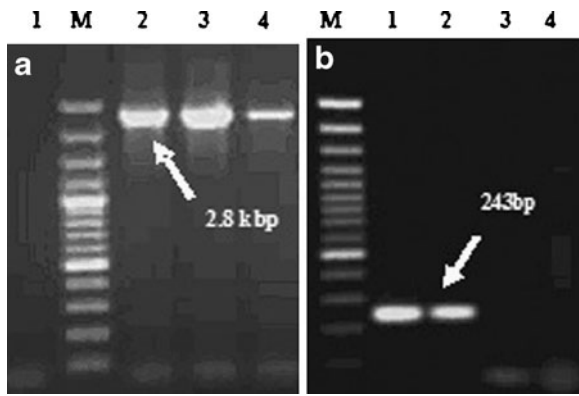


Fig. 2 Agarose gel electrophoresis of PCR products. **a** A-type inclusion gene: DNA from purified GTPV (*lane 1*), purified CMLV 1 (*lane 2*), infected Vero cell lysate of CMLV 2 (*lane 3*), and CMLV-Hyd (*lane 4*), 100-bp DNA ladder plus marker (*lane M*). **b** C18L gene: *lanes 1–4*: CMLV 1, CMLV 2, standard BPXV-BP4, and GTPV, respectively

isolates intradermally (0.25 ml), were observed for mortality, if any as per standard protocols (Munz et al. 1997).

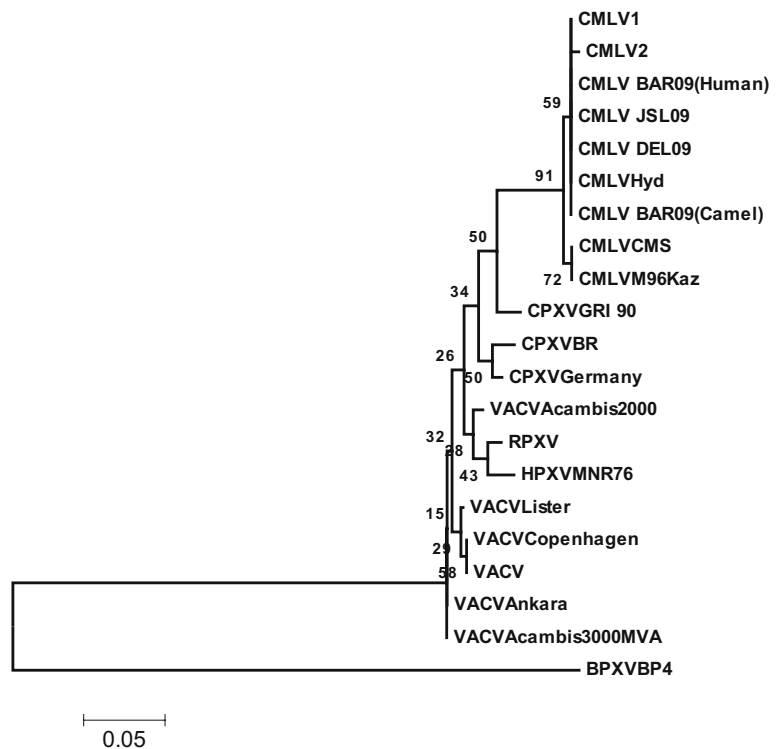
The total genomic DNA was extracted either from scab homogenate or infected Vero cell culture fluid using AuPreP™ DNA Extraction Kit [M/s Life Technologies (India) Pvt Ltd, New Delhi, India] as per manufacturer’s protocol, and finally DNA was eluted in a 50-µl volume. Then DNA was subjected to OPXV-specific PCR for amplification of fragment of ATI gene, employing reported

primers (CoPV01 and CoPV02) (Meyer and Rziha 1993) using long-range PCR enzyme mix (M/s MBI, Fermentas). In addition, to amplify B5R gene, specific PCR cycling conditions were followed as described elsewhere (Balamurugan et al. 2008). Further, diagnostic PCR based on C18L gene in conventional and real-time formats was performed for confirmation (Balamurugan et al. 2009). An aliquot (5 µl) of PCR product was analyzed in 1% to 1.5% agarose gel to visualize the amplicons. The partial C18L gene of three isolates were amplified by PCR and sequenced for authenticity. Phylogenetic tree was constructed based on deduced aa sequences from partial C18L gene sequences by using MEGA 4 program (Tamura et al. 2007).

Results

The CPE started appearing within 36 h post-inoculation during the first passage and was characterized by rounding and ballooning of cells with the formation of micro-plaques (plaque-type) and syncytia (Fig. 1) as reported for other OPXVs. Experimental pathogenesis of the CMLV isolates in rabbits and mice was unsuccessful upon three consequent experiments. The full-length ATI gene-based PCR yielded an amplicon size of ≈2.8 kbp product in all the three isolates (Fig. 2a) confirming the CMLV. The B5R gene was amplified using DNA isolated from three CMLV isolates to yield a product size of 1,019 bp (data not shown). Similarly

Fig. 3 Phylogenetic clustering of CMLVs and other OPXVs constructed based on the deduced aa sequence of C18L gene by interior branch bootstrap test neighbor-joining method using 1,000 replicates of the data set. The bootstrap confidence values of major clusters are indicated in the node of tree. The *bar* represents the genetic distance



as anticipated, diagnostic PCR yielded an amplicon of 243 bp in all the CMLV isolates, whereas no such amplification was noticed in control DNA samples (Fig. 2b). On sequence analysis of C18L gene, all three CMLV isolates showed 99.3–99.6% and 98.7–99.3% identity among themselves at the nucleotide and amino acid levels, respectively. Phylogenetic tree was constructed along with available isolates/strains and analyzed. In the un-rooted tree (Fig. 3) constructed, all the Indian CMLV isolates were clustered together, including the isolates of the present study with other strains (CMLV-CMS and CMLV-M 96 Kazakhstan) of CMLVs.

Discussion

The isolates produced characteristic CPE in Vero cells. Similar type of CPE in Vero cells has been described for CMLV isolates from Asia and Africa (Renner-Muller et al. 1995; Marodam et al. 2006; Al-Ziabi et al. 2007). Failure to reproduce camel pox disease in cattle, sheep, goats, rabbits, guinea pigs, rats, hamsters, and mice has also been reported earlier and this further supports that the CMLV is host specific (Ramyar and Hessami 1972) and may not have public health significance as was reported earlier (Kriz 1982). However, recently, Bera et al. (2010) reported three human cases with involvement of camel pox in camel during 2009 in India, which showed the probable changing pattern of host specificity of virus with decrease in the cohort immunity against OPXV in humans, which could be of serious public health concern. OPXV-PCR based on various genes such as hemagglutinin (HA) and tumor necrosis factor-binding protein receptor-II has earlier been reported for suspected isolates of CMLV (Pfeffer et al. 1996; Al-Ziabi et al. 2007). Although the ATI and the HA gene-based PCRs developed for the diagnosis and differentiation of OPXVs (Meyer and Rziha 1993; Pfeffer et al. 1996) have been effective, these assays may not be suitable for rapid, routine, specific diagnosis of camel pox infections and their simultaneous differentiation (Balamurugan et al. 2009), but in this study the Vero cell-adapted field CMLV isolates were initially identified by inclusion gene-based PCR using Co PV1 and Co PV2, followed by B5R gene-based PCR as reported earlier and later confirmed by C18L gene-based CMLV-specific PCR (Balamurugan et al. 2009). Further, the sequence analyses of partial C18L gene of all the three isolates with other OPXVs showed a high similarity among themselves at the nucleotide and amino acid levels as reported earlier, which revealed a closer identity of Indian isolates of CMLV with reported strains/isolates. Phylogenetic analysis showed that CMLV sequences cluster in a separate group and that these differ from

other OPXV members and share closer relationship with variola virus as reported earlier (Balamurugan et al. 2008).

In conclusion, virus isolation, experimental animal pathogenesis, various gene-based PCRs, and genetic analyses clearly confirmed that the outbreaks were caused by CMLV and identity of CMLV isolates. Further, phylogenetic analyses of partial C18L gene sequences have shown that Indian CMLVs are clustered together with other reported isolates. The first report of camel pox in Indian subcontinent during 1909 and later in neighboring countries warrants biosecurity and biosafety measures especially at borders to contain this transboundary and emerging disease. The control and eradication of sporadic cases of camel pox infection in camel husbandry is of prime importance in India. In this direction, attempts have been made in our laboratory to develop indigenous live attenuated camel pox vaccine using standard CMLV 1 isolate in the direction of a vaccine developed in Saudi Arabia (Hafez et al. 1992). Further, considering the increased incidence of camel pox not only in camel but also likely in human in India, studies on molecular epidemiology, specific diagnosis, and control measures have paramount importance in reducing the circulation of CMLV in camels, and much emphasis should also be given as it is of public health importance.

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