Pathological and molecular investigations of systemic form of camelpox in naturally infected adult male dromedary camels in India

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ABSTRACT

Camelpox is a wide-spread infectious viral disease of camelids. An outbreak of camelpox was reported in 15 adult male dromedary camels aged between 10 to 16 years of an organized herd in winter season. The infected camels showed clinical signs of fever, anorexia, lachrymation, pendulous lips, excessive salivation and pock lesions on the skin of head, neck, mouth, lips, extremities, thigh, abdomen, scrotum and inguinal region. Mortalities were recorded in three infected camels after 10–12 days of infection and showed systemic pox lesions characterized by vesicles, papules, ulcerations and raised pock lesions in the mucous membranes of the mouth, tongue, tracheal mucosa, lung, abomasum and liver. Histopathology study revealed characteristic pox lesions with intra-cytoplasmic eosinophilic inclusion bodies in tongue. Lung showed lesion of interstitial pneumonia (n = 2) and bronchointerstitial pneumonia (n = 1). Liver showed infiltration of mononuclear cells around central veins and degenerative changes of hepatocytes. The abomasum and intestine showed ulcerations, marked capillary congestion and areas of lymphocyte infiltration in mucosa and submucosa. The presence of camelpox virus (CMLV) was confirmed in viral DNA isolated from formalin fixed paraffin embedded (FFPE) tissues of tongue, lung, abomasum, liver, heart and intestine of infected camels by C18L gene PCR. The sequencing of viral DNAs showed phylogenetic relatedness with other CMLV isolates from India and other countries. Thus, our study confirmed the rare severe form of systemic camelpox outbreak in adult male dromedary camels hence future attention should be given for studies on virulence, strain identification and molecular epidemiology of CMLV for planning of effective preventive and control strategies.

1. Introduction

Camels are the most capable animal species in utilizing marginal areas and in survival and production under harsh environmental conditions (Schwartz, 1992). The camel is a very useful animal for desert people living in hot arid Thar region of India, utilized mainly for ploughing, transport, drawing water from deep wells and also provides milk, manure, leather and wool/fur with minimal input. Besides, the camel has also played a significant role in civil law and order, defense and battles from the ancient times in India. Presently, the camel corps constitutes an important wing of Border Security Force (BSF) of Indian Para-Military Services where they are mainly used for patrolling of international border in Rajasthan. Camel is generally considered resistant animal and suffers only few diseases compare to other livestock species. Among such diseases, the occurrence of camelpox is socio-economically significant as it incurs considerable loss in terms of morbidity, mortality, abortion, loss of weight and reduction in milk yield (Bhanuprakash et al., 2010). The outbreaks of camelpox cause enormous economic losses and warrant necessary quarantine and containment measures to prevent the spread of the disease to other countries (Wernery and Zachariah, 1999). Camelpox has been designated as very contagious and extremely transmissible skin disease of camels, which may produce mild skin lesions or severe systemic infections depending upon the virus strain and the immune status of the animal (Wernery and Kaaden, 2002; Dahiya et al., 2016). The disease in mild form is mainly characterized by skin lesions which initially appear as erythematous macules, developing into papules and vesicles, and later turning into pustules. The generalized/systemic form is rare and reported in young animals aged 2–3 years in herds...
associated with weaning and poor nutrition (Wernery and Kaaden, 2002). In systemic form in addition to the skin lesions, the affected camels show pox lesions in the mucous membranes of the mouth and respiratory tract and mortality due to secondary infections and septicaemia (Wernery and Kaaden, 2002; OIE, 2018).

The camelpox is transmitted by either direct or indirect contact via a contaminated environment. The direct transmission occurs in between infected and susceptible animals either by inhalation or through skin abrasion; although a mechanical transmission may play a role (Wernery and Kaaden, 2002). The affected camels may shed the virus through scab materials and secretions like milk, saliva and ocular and nasal discharges in the environment such as in water which becomes then the source of infection for susceptible animals (Wernery and Kaaden, 2002; Khalafalla and Ali, 2007). The virus may survive in the dried scabs for as long as four months.

The etiological agent of camelpox is camelpox virus (CMLV) an epitheliotropic DNA virus classified under the genus Orthopoxvirus (OPXV) of the subfamily Chordopoxvirinae in the Poxviridae family (Essbauer et al., 2009). CMLV is the most closely related to the variola virus, the etiological agent of smallpox and is also considered as an emerging public health problem due to its zoonotic nature and increased reported cases and outbreaks throughout the world (Bera et al., 2011; Balamurugan et al., 2013; Khalafalla and Abdelazim, 2017; Mohammadpour et al., 2020). The World Organization for Animal Health (OIE), lists camelpox as a reportable disease (OIE, 2014). The close similarity of CMLV to variola virus has also raised concerns of its potential use for biological warfare (Nagarajan et al., 2013). Therefore reporting and investigating the camelpox disease outbreaks are very much important for studying the epidemiology of the virus and planning of preventive and control strategies for the eradication of this economically important camel disease. Although vaccination could be the most economical and effective method for control and eradication of camelpox, non availability of commercial vaccines in many camel rearing countries is the major constraint for control of camelpox.

The incidence of camelpox has been frequently reported from all the camel rearing countries of the world especially the developing countries of Asia and Africa in the recent past (Mohammadpour et al., 2020). In India, camelpox is an endemic disease and sporadic outbreaks often reported among camel population of organized and unorganized herds in which they are maintained in a semi intensive system or in open grazing areas by pastoralists mainly from North West part (Rajasthan) of India (Balamurugan et al., 2009; Bhanuprakash et al., 2010; Nagarajan et al., 2013; Kachhawaha et al., 2014; Dahiya et al., 2017; Narnaware et al., 2018). However, since most of the camel farmers in India are nomadic and there is lack of diagnostic facilities at many of the remote areas along their tracks, reporting and regular documentation of morbidity and mortality data of camelpox is not possible (Dahiya et al., 2017). This is one of the major obstacles in combating camelpox in developing countries.

In contrast to the very common systemic form of sheep pox and goat pox, the systemic form of camelpox appears to be rare with limited information on its pathology. The studies on pathology of systemic form of camelpox will help clinicians formulating effective treatment and control measures and preventing mortality. In this study we investigated the pathology of systemic form of camelpox in an outbreak in adult male dromedary camels and also detected CMLV genome in formalin-fixed paraffin-embedded (FFPE) tissues from internal organs of infected camels by PCR followed by a partial gene sequencing and genetic analysis.

2. Material and methods
2.1. Animals and pathological investigations

The dromedary camels of the present study belonged to an organized camel herd of Border Security Force (BSF), Rajasthan, India consisting of 100 adult (aged 10–16 years) male dromedary camels, which were transported to New Delhi in November 2018 and kept in a temporary camp. Among these camels, skin lesions resembling camelpox were observed in total 15 camels with subsequent mortality in three camels. The necropsy was performed by veterinarians from BSF and National Research Centre on Camel, Bikaner. The gross pathological lesions were noted during necropsy examination and tissue samples such as tongue, lung, liver, heart, kidney, intestine and abomasum were collected in 10% formal saline for histopathology. These samples were transported to pathology laboratory of National Research Centre on Camel, Bikaner for further processing and examination. The formalin fixed tissue samples were embedded in paraffin, cut into 4–5 micron sections using a semi automatic microtome (Wewok®, India) and stained with hematoxylin and eosin (HE) stain using the standard procedure (Luna, 1968). The histopathological lesions in HE-stained tissue samples were interpreted by S. D. Narnaware by observing under low (100X, 200X) and high power (400X) of an optical microscope with attached digital camera (Lx500, Labomed®, USA), and noting and imaging any inflammatory or degenerative or vascular changes and also assessing severity and nature of cellular infiltration. All the samples were collected in accordance with the procedures approved by Institutional Animal Ethics Committee of ICAR- National Research Centre on Camel, Bikaner, India (Approval No. 25/19/2018-CPCSEA) and following established animal welfare guidelines.

2.2. DNA extraction and PCR

Since camels of the present outbreak were in a temporary camp where sampling facilities in cold chain were not available hence fresh tissue samples from necropsied camels could not be collected for DNA extraction. The FFPE tissues from lung, tongue, abomasum, liver, heart and intestine of all three cases were processed for genomic DNA extraction using ReliaPrepTM FFPE gDNA Miniprep System (Promega, USA). In order to prevent carryover of contaminating DNA, a fresh sterilized blade was used for each tissue sample. All the extracted DNA were used to amplify C18L gene of CMLV by PCR as described by Balamurugan et al. (2009). To rule out the possibility of infection with contagious ecthyma virus we tested all the extracted DNAs with a parapoxvirus-specific PCR targeting the topoisomerase gene (Nagarajan et al., 2011). The PCR amplified products were visualized in 1% agarose gel and the purified PCR products were subjected to nucleotide sequencing for C18L gene using Sanger sequencing based on the chain-terminating dideoxynucleotides method (Eurofins, India). These sequences obtained were deposited in NCBI GenBank (accession numbers: MT702755 to MT702760) and aligned with the 34 published sequences from India and other countries to compare phylogenetic relatedness. The sequences were aligned by ClustalW tool and the phylogenetic analysis of the nucleotide sequence of the C18L gene of camelpox was inferred using the Maximum Composite Likelihood method (Tamura et al., 2004). This analysis involved 40 nucleotide sequences and the evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).

3. Results
3.1. Clinical signs and gross lesions

In the present study, a total of 15 adult male dromedary camels having age ranging from 10 to 16 years were found affected by camelpox. The affected camels showed initial signs of fever (n = 9; 60%), anorexia (n = 8; 53.33%), lachrymation (n = 11; 73.33%), pendulous lips (n = 10; 66.66%), excessive salivation (n = 12; 80%) and later on poplar and pustular lesions on skin of head, neck, mouth, lips, inguinal region, scrotum, prepuce, abdomen, base of the tail, thighs and legs (n = 15; 100%) (Supplementary Fig. S1). These skin lesions after about one week became dry, scaly, ulcerated and left red and bleeding areas. Out of these 15 aforementioned animals, mortalities
were reported in three camels after 10–12 days of showing symptoms, despite undergoing treatment. These camels, in addition to skin lesions, also had multiple papular, erosive, ulcerative and vesicular lesions inside oral cavity involving mouth, lips, tongue and buccal mucosa (Figure 1A) leading to difficulty in swallowing and restriction of the animals from eating. The necropsy examination of these camels showed poor body condition, mucous discharge from nasal cavity and congestion and froth in tracheal mucosa. The gross lesions in internal organs of these affected camels were more or less similar with varying intensity. The lung of carcass 1 was diffusely enlarged, dark red, wet with presence of frothy exudate in cut surfaces, failed to collapse and had multifocal raised pock lesions having size ranging from 5-15 mm in diameter with some lesions having hemorrhagic or ulcerated centre distributed throughout the lobes but particularly in the caudal lobes (Figure 1B). The lungs of case 2 and 3 also showed similar lesions as in case 1 with presence of multiple raised pock lesions, however in cut surfaces no evidence of exudates found (Figure 1C). The liver of all infected camels showed multiple pale areas of necrosis, mild to moderate congestion and minute raised pock lesions having size ranging from 5-10 mm in diameter (Figure 1 D). The heart showed petechial hemorrhages on epicardium in two cases whereas kidneys showed diffuse congestion and multifocal pale necrotic areas in one case. The abomasal mucosa showed severe congestion, thickening and multiple pock lesions and ulcerations in all infected cases (Figure 1E).

3.2. Histopathological lesions

The histopathology of tongue showed marked epithelial hyperplasia with desquamation and disintegration of mucosal epithelium and multiple papular and vesicular lesions. The epithelial cells forming these lesions showed marked vacuolation and hydropic/ballooning degeneration with presence of characteristic round to oval shaped intracytoplasmic eosinophilic inclusion bodies in large numbers (Figures 2 and 3). Focal areas of mononuclear cellular infiltration consisting of lymphocytes and occasional macrophages and hyperemic blood vessels were also observed in mucosal and submucosal layer.

Histopathology of lung of case 1 showed lesions suggestive of bronchointerstitial pneumonia characterized by thickened alveolar and bronchial walls with infiltration of mixed population of lymphocytes, neutrophils, occasional macrophages, mild fibrous tissue proliferation and also showed fibrinous exudates mixed with necrotic cellular debris (Figure 4). In some areas hyperplasia of bronchial and bronchiolar epithelium with peribronchial infiltration of lymphocytes and occasional macrophages was observed (Figure 5). In some areas the thick and

Figure 1. A. Multiple erosive, ulcerative and vesicular lesions (arrow) in the mucosa of tongue (case 1). B. Multiple pock lesions with hemorrhagic (white arrow) and ulcerated (black arrow) centre on lung surface (case 1). C. Multiple raised pock lesions (arrow) on lung surface (case 2). D. Liver showing minute pock lesions (arrow) and pale areas of necrosis. E. Abomasum showing thickened congested mucosa (white arrow) with ulcerations and pock lesions (black arrow) (case 3).
proliferating bronchial epithelium caused occlusion of small bronchiolar airways leaving only a small lumen for the passage of air. The pleura covering the lung parenchyma was thickened by presence of fibrous tissue proliferation, mononuclear cellular infiltration, hyperplasia of serosal cells and hyperemic blood vessels. Histopathology of lung of case 2 and 3 showed lesions suggestive of interstitial pneumonia characterized by thickened alveolar and bronchial wall due to infiltration of lymphocytes, occasional macrophages, mild fibrous tissue, hyperemic alveolar capillaries and few of the alveoli showing hyperplasia of type II pneumocyte. There was no exudate in alveolar spaces and airways. Marked congestion and perivascular infiltration of lymphocytes and macrophages with anthracosis was observed around majority of the blood vessels and alveolar capillaries (Figure 6). The histology of grossly visible raised pock lesions on lung surface showed thick hyaline membrane composed of dense fibrous connective tissue, flattened epithelial cells and hyperemic blood capillaries which were surrounded by lymphocyte infiltration (Figure 7). These pustular lesion contained fibrinous proteinaceous fluid mixed with cellular debris.

Histopathology of liver of affected camels showed necrosis and vacuolar degenerative changes in hepatocytes, hyperemic sinusoidal capillaries, mild hemosiderosis and infiltration of lymphocytes around central veins (Supplementary Fig. S2). The kidney showed generalized congestion of blood capillaries and multifocal areas of lymphocyte infiltration in intertubular spaces. The size of glomeruli were enlarged with glomerular tufts showing marked increase in cellularity and hyperemia of glomerular capillaries (Supplementary Fig. S3). The abomasum showed hyperplasia, edema and desquamation of mucosal epithelium with vacuolar degenerative changes in epithelial cells (Figure 8). In addition, hyperemic blood vessels and multifocal areas of lymphocyte infiltration in mucosal and submucosal region was also observed. Histopathology of heart showed generalized vascular congestion and focal areas of necrosis and degeneration of cardiac muscles (Supplementary Fig. S4). The small intestine showed short, broad and fused villi with infiltration of lymphocytes and eosinophils in lamina propria and crypt region, and marked congestion of blood vessels in mucosa and submucosa (Figure 9).

### 3.3. Molecular diagnosis

The molecular diagnosis of the camelpox using PCR revealed amplification of C18L gene of CMLV from DNA extracted from FFPE tissues from internal organs such as lung, tongue and liver of all the three infected camels showing expected amplicon size of 243 bp (Supplementary Fig. S5). Whereas PCR of FFPE tissues from abomasum, heart
and intestine were found positive in two infected camels. All the above DNA samples found negative for amplification of topoisomerase gene of contagious ecthyma used for differential diagnosis. The phylogenetic analysis showed that all the sequences in the present study clustered with the reference camelpox sequence (Figure 10).

4. Discussion

There are very few reports on systemic form of camelpox infection with involvement of internal organs and detection of CMLV or its genome from visceral organs of affected dromedary camels. In this study we investigated pathological lesions in systemic form of camelpox infection and also detected the presence of CMLV genome from major internal organs of acutely infected adult male dromedary camels. The findings of this study first time show that adult camels are equally susceptible to severe form of systemic camelpox disease. This finding is contrary to the general belief that severe and systemic form of camelpox mostly affects young camels (2–3 years old) and the disease is milder in old camels (Wernery and Kaaden, 2002; Bayisa 2019; Balamurugan et al., 2009). The outbreaks of camelpox with more severe form were mostly reported during the rainy season in endemic countries (Mayer and Czerny, 1990; Wernery et al., 1997; Wernery and Kaaden, 2002; Bayisa, 2019; Kachhawaha et al., 2014). However the present and previous outbreaks reported in recent past from India showed the disease incidence in winter season (Dahiya et al., 2017; Narnaware et al., 2018). The risk factors associated with higher incidence of camelpox in previous studies includes young age, stress, nutritional status of the animals, rainy season, migration of animals, common watering, presence of other diseases in incubation phase and the virulence of the virus (Alhendi et al., 1994; Nothelfer et al., 1995; Wernery and Kaaden, 2002; Khalafalla and Ali, 2007; Bayisa, 2019; Balamurugan et al., 2013). Therefore in order to reduce the impact of the disease in endemic areas, community awareness on these risk factors is required.

The total incidence of camelpox in the affected herd was recorded as 15% which is comparable with incidence in previous studies from India and other countries (Narnaware et al., 2018; Mohammadjour et al., 2020). The mortality rate (3%) of the present study was also comparable with studies reporting mortality between 5% to 28% in adult camels (Balamurugan et al., 2013), whereas in young animals the mortality was recorded as high as 25%–100% (Mayer and Czerny, 1990). The mortality in camelpox is usually influenced by the presence of inter-current diseases (notably trypanosomiasis), stress, age, and the nutritional status of the animal and virus virulence (Alhendi et al., 1994). In the present outbreak, typical symptoms and lesions of camel pox such as fever,

Figure 6. Histopathology of lung showing thickened alveolar septa due to lymphocyte infiltration and hyperemic alveolar capillaries. Also note perivascular infiltration of lymphocytes and macrophages (case 2). HE X 200.

Figure 7. Histopathology of pock lesions in lung showing thick hyaline membrane (arrow) composed of dense fibrous connective tissue, flattened epithelial cells and hyperemic blood capillaries which were surrounded by lymphocyte infiltration (case 2). HE X 200.

Figure 8. Histopathology of abomasum showing necrotic and desquamated mucosal epithelium along with hyperemic blood vessels. HE × 400.

Figure 9. Histopathology of small intestine showing short, broad and fused villi with infiltration of lymphocytes and eosinophils. Also note marked capillary congestion in mucosa. HE × 200.
lachrymation, excessive salivation and pustular skin lesions were observed (Aregawi and Feyissa, 2016; Dahiya et al., 2017; Narnaware et al., 2018). However mortalities due to camelpox were reported in only few of the previous studies which were published almost 20 years before and since then no systemic camelpox disease was reported across the world (Wernery et al., 1997; Kinne et al., 1998; Pfeffer et al., 1998; Wernery and Zachariah, 1999). This variation in disease manifestation may be attributed to the difference in virulence of the infecting virus strain and also the immune status of animals (Wernery and Kaaden, 2002). Since the infected camels of the present study had history of recent transportation from different parts of Rajasthan to New Delhi, before the outbreak, hence there is possibility that the infected camels may have compromised immune status due to transportation stress.

There is very limited information on the pathology of systemic form of camelpox. Multiple pox-like lesions on the mucous membranes of the mouth, respiratory and digestive tracts along with infection of the heart and liver were observed in fatal form of camelpox infection in previous studies (Kinne et al., 1998; Pfeffer et al., 1998; Wernery and Zachariah, 1999). This variation in disease manifestation may be attributed to the difference in virulence of the infecting virus strain and also the immune status of animals (Wernery and Kaaden, 2002). Since the infected camels of the present study had history of recent transportation from different parts of Rajasthan to New Delhi, before the outbreak, hence there is possibility that the infected camels may have compromised immune status due to transportation stress.

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A PCR assay targeting the encoding ankyrin repeat protein (C18L gene) which specifically identify CMLV and differentiate it from other OPXVs, capripoxviruses and parapoxviruses has been developed with the sensitivity limit as low as 0.4 ng of viral DNA in CMLV suspected cases (Balamurugan et al., 2009). Although the AT1, hemagglutinin (HA) and DNA polymerase (DNA Pol) gene-based PCRs developed for the diagnosis and differentiation of OPXVs (Meyer and Rziha, 1993; Dahiya et al., 2017) have been effective, these assays may not be suitable for rapid, routine, specific diagnosis of camelpox infections and their simultaneous differentiation due to less sensitivity related to PCR product size (Balamurugan et al., 2009). The amplification of C18L gene from virus DNA obtained from FFPE tissue samples of the present study also substantiate the usefulness and sensitivity of this PCR assay for rapid and specific identification of CMLV which also signifies its role in diagnostic PCR. However PCR-based analyses of DNA extracted from formalin-fixed materials face challenges mainly due to fragmentation of the DNA and crosslinked protein-DNA complexes besides induction of N-hydroxymethyl mono-adducts on guanine, adenine and cytosine and Nmethylene crosslinks between adjacent purines in DNA (Yun et al., 2018). Therefore instead of full HA and partial A-type inclusion protein (ATIP) genes of CMLV, C18L gene of CMLV was used for amplification from FFPE tissues of the present study. For histopathologic investigations, tissue samples are mostly stored as formalin-fixed, paraffin-embedded blocks. The application of PCR from FFPE tissues could not only widen the scope of routine diagnosis but also helps in studies on molecular epidemiology of CMLV from archival tissue samples.

It is believed that different strains of camelpox may have difference in their virulence and may be responsible for causing two forms of the disease i.e. local and generalized, but this has never been demonstrated (Wernery and Zachariah, 1999; Nagarakaran et al., 2013). Moreover as such no detail information is available regarding CMLV strains circulating in India and other countries inspire of regular outbreaks (Wernery and Kaaden, 2002; Dahiya et al., 2017). In this context, further sequencing of CMLV strains isolated from mild localized and severe generalized forms of camelpox cases could further explore discrepancies in their gene contents and allow the identification of genes associated with virulence or immune-evasion properties (Duraffour et al., 2011). Moreover, due to the sporadic reports on zoonotic cases of camelpox infection (Bera et al., 2011; Khailafla and Abdelazim, 2017; Mohamadpour et al., 2020), it is very essential to study the virulence of CMLV circulating in different parts of the world. Hence, the CMLV strains identified from each and every outbreak needs further molecular characterization for exploring its host range, host response to infection and virulence properties which will also form the basis for development of future vaccines and control strategies. It was found that the detected CMLV DNAs from three camels of the present study shared sequence homology (100%) with each other and also with previously reported strains from India wherein only mild localized or skin lesions were reported (Dahiya et al., 2017), except one isolate i.e. MT702759 which has amino acid isoleucine and threonine at position 14 and 24 from N terminal end respectively whereas all other isolates has leucine and arginine in that place. Since the present study involves partial sequencing of the isolates intended for diagnostic purpose, a full length gene sequencing would be helpful to deduce information about molecular difference between strains with different clinical manifestation of the disease i.e. cutaneous or systemic form.

5. Conclusion

The present study reported significant pathological findings along with detection of CMLV genome in internal organs of systemic form of camelpox infected adult male dromedary camels which are rarely documented. Considering the increased reported cases and number of outbreaks of camelpox from India and other camel rearing developing countries, future attention should be given to understand its transmission chain, molecular epidemiology, improved diagnostic methods and adoption of appropriate prophylaxis and control measures for control and eradication of this economically important camel disease.

Declarations

Author contribution statement

Shirish Dadarao Narnaware, Rakesh Ranjan, Shyam Singh Dahiya: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Amar Panchbudhde, Devika Bajpai: Performed the experiments; Analyzed and interpreted the data.

Fateh Chand Tuteja, Rajesh Kumar Sawal: Contributed reagents, materials, analysis tools or data.

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Data availability statement

Data included in article supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

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