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Molecular detection of lumpy skin disease virus from Tamil Nadu, India

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Abstract
Lumpy skin disease (LSD) is an emerging, OIE notifiable pox virus disease affecting cattle and buffalo in India. It is caused by LSD virus that shares the genetic relatedness with sheeppox and goatpox viruses in the genus *Capripox virus.* The current investigation deals with molecular detection of LSD infection in cattle of Tamil Nadu, a southern state of India during the period from August 2020 to December 2021. The affected cattle showed generalized nodular lesions and other clinical signs suggestive of LSD. A total of 104 samples including 81 scab tissues and 23 whole blood samples were collected and subjected to molecular PCR specific for capripox viruses. Of which, 78 samples including 76 scab and 2 positive blood samples were found positive by PCR to yield 192 bp product. The report supports the use of molecular PCR for direct detection of LSD in cattle from field samples and hence the disease can be differentiated from similar clinical conditions in cattle.

Keywords: Lumpy skin disease, cattle, capripox virus, PCR

1. Introduction
Lumpy skin disease (LSD) is an OIE notifiable, high-impact transboundary poxviral disease of cattle and Asian water buffalo. It affects the cattle of all ages and breeds (Saegerman *et al*., 2018) [6]. LSD is caused by LSD virus of the genus *capripoxvirus*, belonging to subfamily *Chordopoxvirniae* within the family *Poxviridae* (Buller *et al.*, 2005). Other members of genus include sheeppox virus (SPPV) and goatpox virus (GTPV) affecting sheep and goat, respectively.
The disease is host-specific and transmitted predominantly by arthropod vectors mechanically. Though morbidity is relatively high upto 100%, the mortality is usually low (Abutarbush *et al*. 2013) [7] less than 1-5%. The clinical severity of the infection varies depending upon the circulating LSDV strain, vector prevalence, age and immune status of the host animal (Tuppurainen *et al*., 2017) [8]. The clinical signs include fever for initial 1-3 days, followed by rhinitis, lachrymation, enlargement of lymph nodes, anorexia, dysgalactiae, depression and disinclination to move (Tasioudi *et al*., 2016) [9]. The skin nodules (0.5-5.0 cm size) appear within 1-2 days and gradually become harder and necrotic thus inducing severe discomfort, pain and lameness. These nodules can be observed all over the body including head, neck, udder, scrotum, perineum and buccal mucosa (Babiuk *et al*., 2008; Tageldin *et al*., 2014) [11, 10]. They may either regress in 2 or 3 weeks or may results in hard, raised necrotic areas ('sit-fasts') clearly separated from the surrounding skin. This usually becomes liable to myasis or secondary bacterial infection. Further, the affected cows become infertile or abort, if pregnant; the bulls become either temporarily or permanently infertile and secrete the virus for a longer duration.
The disease is economically significant as it causes both tangible losses and intangible losses. Decreased milk yield and meat quality, abortion, infertility, damaged skin and hide, culling of affected cattle comprise tangible losses (Tuppurainen and Oura, 2012) [12]. Whereas restricted cattle movement and trade restriction are intangible losses. The disease can be diagnosed based on clinical signs, however the clinical signs and lesions must be differentiated from pseudo lumpy skin disease, dermatophytosis, dermatophilosis, photosensitisation, insect bites etc. (Tuppurainen *et al*., 2018). The similar confounding skin lesions in cattle necessitate identification of LSD by molecular diagnostic techniques such as conventional gel-based PCR and real-time PCR (Ireland and Binepal, 1998; Tuppurainen *et al*., 2005) [5, 13]. Further, as serological tests are not practicable for differentiation of capripoxviruses, sequencing and

phylogenetic analysis can be done for the differentiation of LSDV.

LSD was first recorded in Zambia in 1929 and historically was restricted to African countries. After 1988-89, it has spread outside sub-Saharan Africa in Egypt and Israel between (OIE, 2017) [14] and subsequently reported from many countries. LSD has further expanded and the first report of LSDV from India is from the state of Odisha in 2019 (Sudhakar *et al*., 2020) [4] followed by reports from other states of India, including Kerala, Tamil Nadu, Andhra Pradesh, Telangana, Jharkhand, West Bengal, Assam, Maharashtra, Chhattisgarh and Madhya Pradesh of central parts of India (Kumar *et al*., 2021) [3]. Recently it is reported from Gujarat and Rajasthan also. The present study describes about the molecular diagnosis of LSDV infection from Tamil Nadu, one of the Southern state of India.

2. Materials and Methods

2.1. Clinical appraisal and collection of samples

The animals with typical nodular lesions suggestive of LSD were reported from many places of Tamil Nadu. Along with skin lesions the animals were reported to be observed with symptoms such as fever, deceased milk yield, anorexia, edema in dependent parts and difficulty in walking. Since, the clinical signs and lesions were suggestive of LSDV; the samples such as scab and whole blood were collected from the affected animals. A total of 104 samples (81 scab tissues and 23 unclotted blood samples) were collected from different places of Tamil Nadu, India during the period from August 2020 to December 2021, the according to established guidelines with due consent from the farmers. The whole blood (3ml in heparin/EDTA tubes) and scabs were transported on ice and stored at 4 ºC till use.

2.2. Samples processing and viral genomic DNA extraction

Scab tissues were cut into small pieces, triturated and a homogenized suspension (10% w/v) was made in sterile PBS (pH 7.4). The tissue homogenates were further freeze-thawed three times and clarified by refrigerated centrifuging at 3000 rpm for 10 min to remove any gross debris. The supernatant containing the virus was stored at -20 ºC till use. The DNA was extracted from unclotted blood and supernatant from scab samples by using DNeasy® Blood & Tissue Kit (Cat. No. 69504; M/s Qiagen, Germany), according to the manufacturer's instructions with minor modifications. In brief, 100µl of blood or scab homogenate was taken as starting material with 3-8 h lysis at 56 ºC and eluted in 50 µl elution buffer provided with the kit and kept at -20 °C for use in PCR.

2.3. Molecular PCR

The isolated DNA was subjected to capripox virus specific P32 (LSDV074) gene-based diagnostic PCR as described earlier (Ireland and Binepal, 1998) [5]. The primers and amplification conditions used are mentioned in Table 1. These amplifies 192 bp fragment of this envelope protein gene. The PCR reactions were performed in a final volume of 25 µl consisting of 12.5 µl of Taq DNA polymerase 2x Master Mix RED (Ampliqon, Denmark) with 1.5mM $MgCl_2$, 10 pmol of each forward and reverse primers, 8.5 µl of nuclease-free water and 2µl of isolated template DNA. The amplification was carried out in a thermal cycler (Eppendorf Mastercycler® nexus GX2, Hamburg, Germany). The amplified PCR products were separated and analyzed by agarose gel electrophoresis on 1.5% gel containing 1 µg/ml of ethidium bromide in 1x TAE buffer.

3. Results and Discussion

Lumpy skin disease is an economically important, emerging and notifiable disease according to World Organisation for Animal Health (OIE). Though, thought to be restricted to Sub-Saharan Africa, the transboundary spread to other countries started after 1988-89 (House *et al*., 1990) [15]. Recently, the disease has been reported as emerging disease in Asian countries such as Bangladesh (Hasib *et al*., 2021) [16], China (Lu *et al*., 2021) [17], India (Sudhakar *et al*., 2020) [4], Nepal (Acharya and Subedi, 2020) [19], Vietnam (Tran *et al*., 2021) [18], Bhutan, Hong Kong (Flannery *et al*., 2021) [20], Myanmar (Maw *et al*., 2022) [21], Thailand (Arjkumpa *et al*., 2022) [24], Malaysia (Khoo *et al*., 2022) [22], Laos and Cambodia (Azeem *et al*., 2022) [23]. Hence, the importance of this disease also increases as the way of control and eradication of LSD is very difficult. In India, the outbreak were reported from many states including Tamil Nadu (Kumar *et al*., 2021; Lakshmi Kavitha *et al*., 2021) [3, 2] and posed a huge economic impact on cattle husbandry.

In the present study, the affected cattle showed initial pyrexia (>40-41 ºC) for a period of 1-4 days with a sudden drop in milk production. The animals showed generalized nodular lesions in various parts of the body (Fig. 1) including head, neck, body, hind quarters, legs and udder. These nodules were of 1-5 cm in size, round, circumscribed, painful to touch and later some of these nodules ruptured to bleed and created a deep-seated wounds, that further were complicated by secondary bacterial infections. In some animals, the nodules resulted in necrosed, hard, raided area (the "sit fasts") (Fig. 2). Further, the animals exhibited edema in the limbs and hence the animals were reluctant to move. The lesions on the fetlock regions were severe, extending up to the underlying subcutaneous region and muscles (Fig. 3). The animal also revealed anorexia, nasal discharge, ocular discharge and enlargement of lymphnodes. The clinical signs were in agreement with the earlier reports suggestive of LSD (Salib and Osman, 2011; Kumar *et al*., 2021) [25, 3]. The disease was reported among both in native breeds (*Bos indicus*) as well as cross-bred (*Bos taurus*) cattle, of which the later is being the most affected as reported by Lakshmi Kavitha *et al*. (2021) [2]. The affected calves showed extensive lesions compared to the adults. Here, could not investigate LSDV infection any male animals regarding the impact of the disease.

The diagnosis of the disease was done by using conventional PCR as described earlier (Ireland and Binepal, 1998) [5]. Out of 104 samples subjected to molecular PCR, 76 scab tissue samples (93.8 per cent positivity) and 2 unclotted blood samples were found positive for capripox virus specific genome that yielded an amplicon size of 192 bp (Fig. 4). Further, the representative samples were confirmed as LSDV by sequencing (data not shown) of the positive PCR products. The collection of blood from animals in non-viraemic stage could be the probable reason for less number of positive results in blood samples. Further, as the virus is having the predilection to skin tissue, the scab samples containing high concentration of virus were found to be the most appropriate for diagnosis of LSD (Zeynalova *et al*., 2016) [26].

It is concluded that the current study confirms the presence of LSD virus by molecular PCR. This would throw light on the widespread occurrence of the disease, thus can be inferred as that there is need for better prevention and control measures

to adopt and need for development of effective vaccines against this economically significant disease to mitigate the loss to the farming community.

Table 1: Details of Primers and PCR cycling conditions used in the study

Gene Target	Orientation	Primer Sequence (5'-……-3')	Cycling conditions	Amplicon Size	Reference
Viral attachment protein (P32)	Forward	TCCGAGCTCTTTCCTGATTTTTCTTACTAT	95 ºC, 3 min; 34 cycles of 95 ºC, 45 sec, 50 ºC for 50 sec, 72 ºC for 1 min; followed by 72 ºC, for 10 min	192 bp	Ireland and Binepal, 1998 [5]
	Reverse	TATGGTACCTAAATTATATACGTAAATAAC			

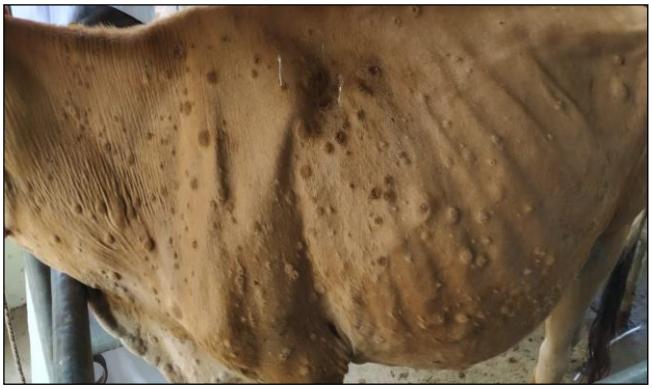

Fig 1: Round, circumscribed nodules (1-5cm) seen all over the body

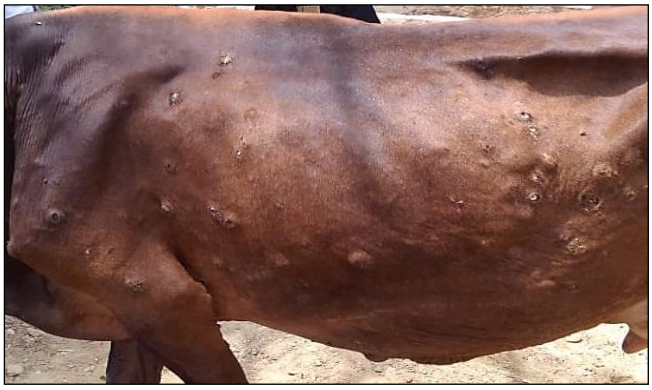

Fig 2: Raised, hard "sit-fasts" lesions

Fig 3: Extensive lesion in the fetlock region affecting the underlying muscle and subcutaneous tissues

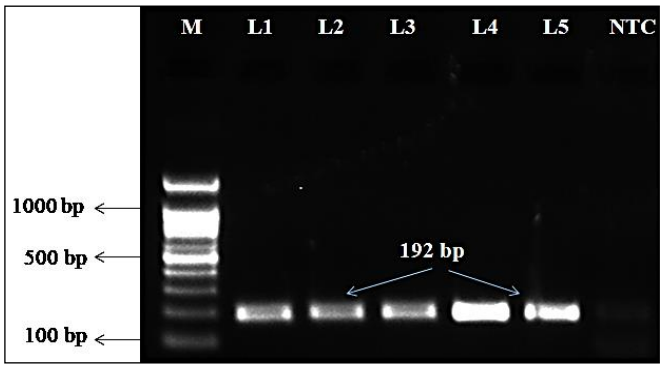

Fig 4: M: 100 bp DNA Marker; L1-L5: Positive Scab samples; NTC: Non-template control

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