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Sanjeeva Kumar M Topan
Assistant Professor, Southern Regional Disease Diagnostic Laboratory (SRDDL) Institute of Animal Health & Veterinary Biologicals (KVAFSU) Hebbal, Bengaluru, Karnataka, India

Shivaraj B Murag
Assistant Professor, Southern Regional Disease Diagnostic Laboratory (SRDDL) Institute of Animal Health & Veterinary Biologicals (KVAFSU) Hebbal, Bengaluru, Karnataka, India

Shivashankar BP
Assistant Professor, Southern Regional Disease Diagnostic Laboratory (SRDDL) Institute of Animal Health & Veterinary Biologicals (KVAFSU) Hebbal, Bengaluru, Karnataka, India

Nandini P
Sr. Technician - SRDDL, Diagnostic Virology Laboratory, Southern Regional Disease Diagnostic Laboratory (SRDDL) Institute of Animal Health & Veterinary Biologicals (KVAFSU) Hebbal, Bengaluru, Karnataka, India

Gowthami CN
Sr. Technician - SRDDL, Diagnostic Virology Laboratory, Southern Regional Disease Diagnostic Laboratory (SRDDL) Institute of Animal Health & Veterinary Biologicals (KVAFSU) Hebbal, Bengaluru, Karnataka, India

K Sripad
Prof and Head-SRDDL, Southern Regional Disease Diagnostic Laboratory (SRDDL) Institute of Animal Health & Veterinary Biologicals (KVAFSU) Hebbal, Bengaluru, Karnataka, India

Venkatesha MD
Director, Southern Regional Disease Diagnostic Laboratory (SRDDL) Institute of Animal Health & Veterinary Biologicals (KVAFSU) Hebbal, Bengaluru, Karnataka, India

Corresponding Author:
Sanjeeva Kumar M Topan
Assistant Professor, Southern Regional Disease Diagnostic Laboratory (SRDDL) Institute of Animal Health & Veterinary Biologicals (KVAFSU) Hebbal, Bengaluru, Karnataka, India

Outbreak investigation and molecular confirmation of lumpy skin disease (LSD) in Karnataka state

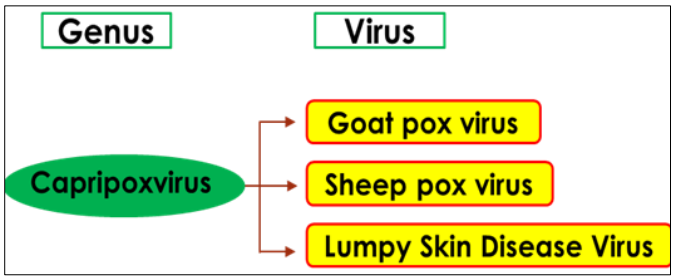
Sanjeeva Kumar M Topan, Shivaraj B Murag, Shivashankar BP, Nandini P, Gowthami CN, K Sripad and Venkatesha MD

Abstract
Lumpy skin disease (LSD) is an economically important WAHO/OIE notifiable transboundary disease of cattle and buffaloes, caused by lumpy skin disease virus, a member of the *Capri poxvirus genus* in the Poxviridae family. The disease is transmitted by arthropod vectors and causes high morbidity and low mortality in infected animals. The susceptible hosts contract the virus mechanically from blood sucking insect vectors and other hematophagous arthropods. Lumpy skin disease was first reported in India during 2019 among cattle in Odisha state. Lumpy skin disease is manifested by distinguishing firm, circumscribed, few to multiple skin nodules or lumps. Present study describes lumpy skin disease outbreak investigations undertaken by the authors in the last two years and molecular confirmation of LSD outbreaks.

Epidemiological and demographic data was collected from the farmer vis-à-vis local Vet during the investigation visit. Affected animals were clinically examined for the signs of the disease and representative clinical samples were collected for laboratory analysis. DNA was isolated from clinical samples and subjected for polymerase chain reaction assay with specific primers targeting conserved gene of lumpy skin disease virus. PCR products resolved on agarose gel yielded a single amplicon of 192 bp confirming specific amplification. LSD positive PCR products were commercially sequenced. Homogeneity analysis of sequence data using BLAST revealed 91.9% match with the lumpy skin disease virus sequences available in the NCBI database. Phylogenetic tree construction of sequences revealed sequence identity to LSDV isolates from Odisha and Bangladesh hence, confirming LSDV infection among affected animals.

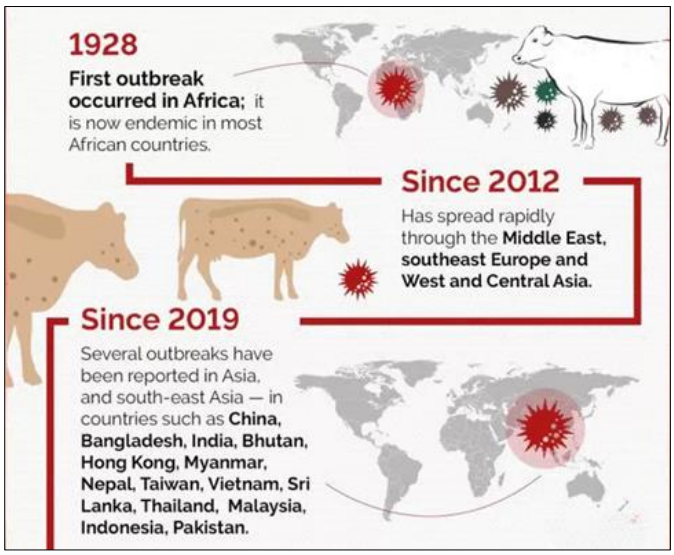
Keywords: Lumpy skin disease virus, Arthropod vectors**,** Morbidity**,** polymerase chain reaction**,** Sequencing, Phylogenetic analysis

Introduction
Lumpy skin disease (LSD) is a highly transmissible transboundary pox viral disease affecting cattle of all ages and breeds (Krešic *et al*., 2020) [3]. However, severity of the clinical signs of LSD varies from subclinical to fatal extent depending on the virulence of the infecting strains and the cattle breed's susceptibility (Lu *et al*., 2021) [5]. Lumpy skin disease virus (LSDV), sheep pox virus (SPV) and goat pox virus (GPV) are the three members in the capripoxvirus (CaPV) genus within the poxviridae family (Fay *et al*., 2020) [1]. The LSD virus has a large double-stranded DNA genome of ~ 151 thousand base pairs (kbps) encoding 156 putative viral genes. LSDV is extremely host specific and under the natural conditions it mainly infects cattle and buffalo. World Organization for Animal Health listed LSD as a notifiable disease due to its significant threat to the cattle population and substantial economic impact. The characteristic clinical feature of Lumpy skin disease is the distinctive and numerous/generalized raised cutaneous nodules/lumps ranging from 0.5 to 5 cm in diameter, that develop over a period of 3 to 4 days from macules to papules to nodules. These cutaneous lesions are frequently accompanied by oral, nasal, and ocular discharge; lethargy; anorexia; and in lactating animals, a rapid drop in milk production. Brisket edema and superficial lymphadenopathy have also been reported. After about 1 to 2 weeks, the skin nodules may resolve over the time or become necrotic, and the center of the nodule eventually sloughs (Sanz-Bernardo *et al*., 2020) [6].



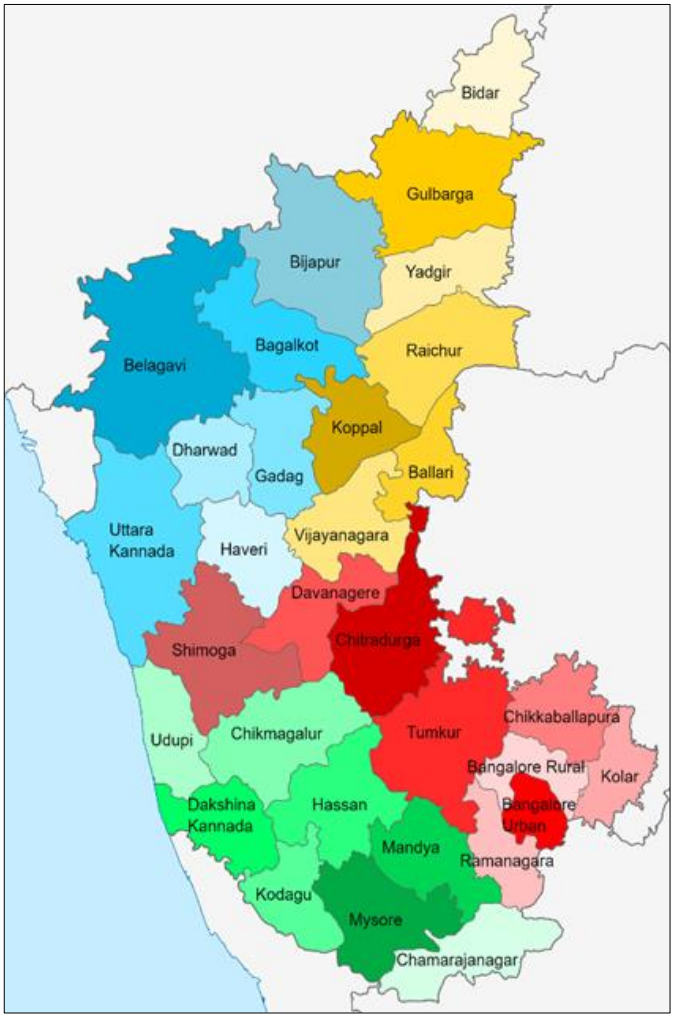
Epidemiology and transmission

LSD was first documented among cattle in Zambia in 1929, subsequently, was limited to sub-Saharan Africa in the succeeding decades. However, LSDV continued to expand in African countries in the recent years. LSDV outbreak outside of the African continent was recorded in Egypt during 1989. Ever since, LSD outbreaks have been frequently reported in Middle Eastern countries and reached Europe in 2013. LSDV spread into east Asia for the first time, and was reported in China, India, and Bangladesh since 2019 (Lu *et al*., 2020; Sudhakar *et al*., 2020) [7]. LSDV in cattle and water buffalo (*Bubalis bubalis*) is transmitted mechanically by various blood-sucking arthropod vectors such as mosquitoes (*Aedes aegypti*), ticks (*Amblyomma hebraeum* and *Rhipicephalus appendiculatus*), and stable flies (*Stomoxys calcitrans*). (Krešic *et al*., 2020) [3]. The morbidity rate varies from 2 to 45% while the mortality rate is usually under 10 percent. Often, water buffalos are less involved and are fairly resistant to the infection when compared to cattle. Cattle with high production stress like milking cows exhibit clinical disease rapidly (Fay *et al*., 2020; Tuppurainen *et al*., 2020) [1, 8].



Lumpy skin disease outbreaks in Karnataka state

In recent years, LSD has been reported from neighbouring countries like China and Bangladesh. The first outbreak of LSD was reported from Odisha during August 2019, in monsoon season with high humidity and vector density. with no mortality and 7.1% morbidity among cattle. Since then, many Indian states have reported LSD outbreaks. Highest number of outbreaks were recorded in 2022 with alarming morbidity and mortality rates in most of the northern states, especially in the states of Rajasthan, Gujarat, Punjab, Haryana and Maharashtra. From Karnataka state, the first samples tested positive for LSD were from Kalaburagi district during 2020.since, then, the disease has spread to most of the districts in the state. It is to be noted that, during, 2020-2021, the outbreaks were restricted to certain pockets or villages in few districts and was a self-limiting disease with nil to very low mortality. By early 2022 all 31 districts have reported LSD outbreaks in the state. However, the LSD outbreak's recorded during 2022, were more aggressive in nature with large morbidity and alarmingly high mortality observed among the affected cattle. All the districts have reported mortality due to LSD, however, Belagavi and Haveri have reported highest mortality. LSD was reported in 4,380 villages of 160 taluks in 31 districts by the end of calendar year 2022. Over a period of time, the disease progression appreciably declined due to effective vaccination coverage and implementation of policies like movement restriction and ban on organisation of sandies/animal fairs etc, establishment of check posts for control of animal movement across the bordering districts/states by the state Government. Although, the reasons for the spread of LSD to India are unknown but it may be due to livestock movement across international borders or may be due to vectors movement from the neighbouring countries. Therefore, understanding the epidemiology of exotic diseases becomes necessary for timely planning the effective disease management strategies.



***Source*:**
https://commons.wikimedia.org/wiki/File:Karnataka_districts_map.svg

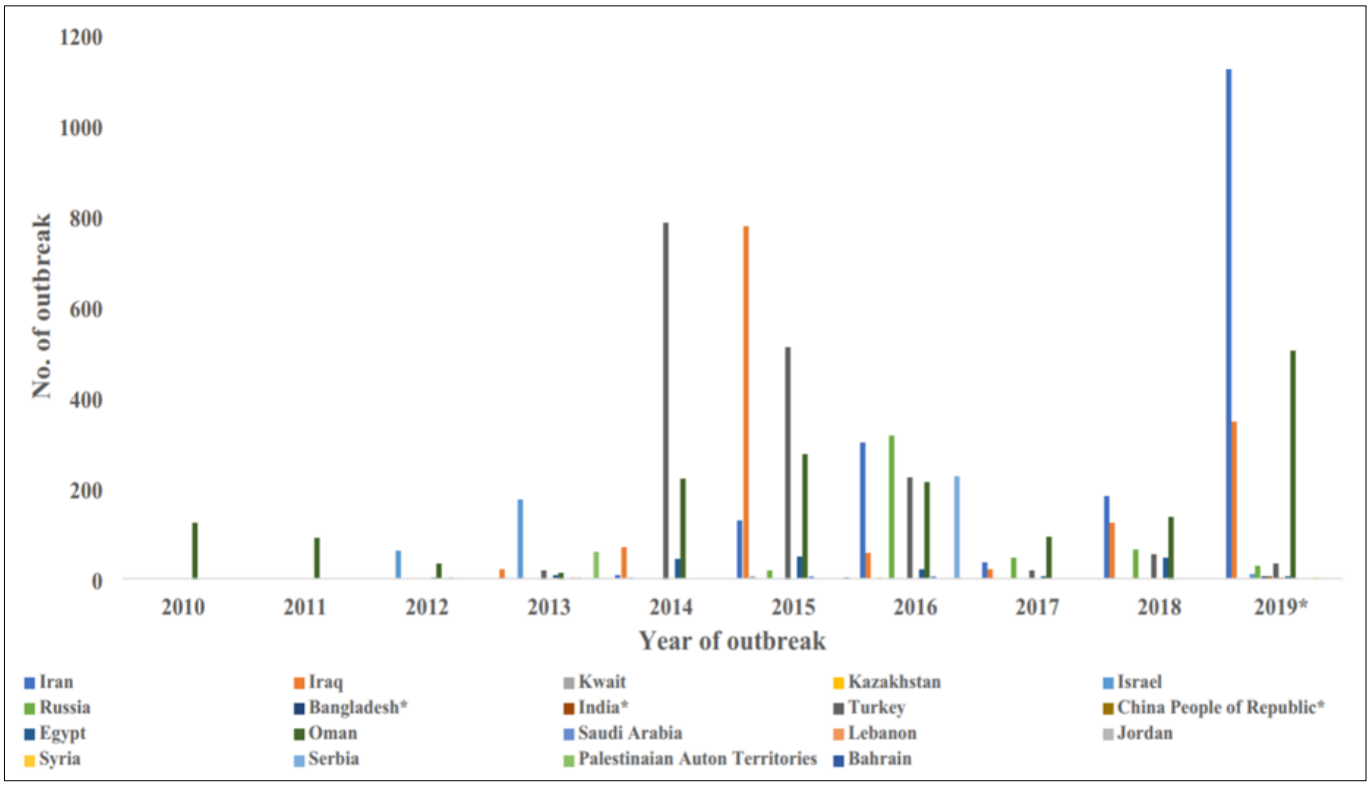


Fig 1: Temporal distribution of LSD virus in Asian countries from 2010 to 2019 (Gupta *et al*., 2020).

Materials and Methods

Outbreak investigation

The present paper describes about the selected disease outbreak investigations conducted in five districts of Karnataka state *viz*: Bangalore Urban, Bangalore Rural, Davanagere, Belagavi and Chikkaballapur districts. The disease investigation team comprising of scientists specialized in veterinary Virology, Bacteriology and pathology visited the affected villages and conducted clinical examination and collected relevant data pertaining to ongoing suspected disease outbreak. The farmer-specialist interactions were focused to explore information on key points like, a) history of the disease course, b) duration of ongoing disease incidence, c) Magnitude of the disease problem: number of cases, population at risk, d) likely, suspicion on sources of infection, d) information on mass movement of animals in the recent past and e) weather data in the geographical setting. Various biological/ clinical samples like Blood/scab tissues/swabs were collected from each investigation visit and transported in cold chain to diagnostic laboratory.

Molecular identification by PCR

The genomic DNA was isolated from clinical samples, using commercially available nucleic acid extraction kit (Himedia) following the protocol described by the manufacturer. Briefly, 200µl blood/swab/scab lysate was taken in 2ml Eppendorf tubes & 20µl of Proteinase K was added and vortexed for 10-15sec. Lysis buffer was added to the sample lysate and vortexed and incubated at 55°C for 10min. The samples were mixed with 200µl of Ethanol (100%) loaded onto the Spin column and centrifuged at 10,000 rpm for 1min, the flow through was discarded. Prewash and wash solution (500µl) was applied on to the spin columns and centrifuged at 10,000 rpm for 1min respectively. Finally, the DNA was eluted in 100µl of Elution buffer. A simple, fast and sensitive conventional gel-based PCR described previously by Tuppurainen *et al*., (2005) [9] was employed in the present study. The conserved PCR primers targeted for amplification of gene encoding the viral attachment protein (LSDV-F-5'-TCCGAGCTCTTTCCTGATTTTTCTTACTAT-3' & LSDV-R-5'-ATGGTACCTAAATTATATACGTAAATAAC-3') yielded an amplicon size of 192 bp. PCR amplification was carried out in a final volume of 25 µl ready to use 2× PCR master mix (Genie) on Takara (Japan) thermal cycler. Thermal profile included initial denaturation at 95 °C for 2 min, and for 35 cycles of denaturation at 95 °C for 45 sec annealing at 55°C for 50 s, and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 2 min. Known positive and negative controls were included. Ten microliters of each amplified product were resolved on 2% agarose (Lonza, USA) by agarose gel electrophoresis using a 100-bp DNA ladder (Genie) and visualised on geldoc system. Two PCR amplicons were gel purified (GCC biotech, India) and sequenced commercially by sanger sequencing, on ABI PRISM system. The obtained nucleotide sequences were analysed using NCBI Basic Local Alignment Search Tool (BLAST) and were aligned with published sequences in GenBank. Sequence analysis was done using MEGA X software and phylogenetic tree was constructed using Neighbour Joining method.

Results

Outbreak investigation

Animal disease outbreak investigation is an art that requires extrapolation of relevant and leading information on suspected ongoing disease outbreak by intuitive interaction with the animal owners vis-à-vis farmers of the affected animals besides technical discussion with the jurisdictional veterinary staff. This interaction is the key for gathering crucial epidemiological data on ongoing disease problem.

During the visit, clinical examination of affected animals was carried out and evident clinical observations like pyrexia, anorexia, drop in milk yield and generalised appearance of nodules/lumps were recorded along with the owner and other relevant demographic and epidemiological data like duration of onset of disease problem, Besides, farmer-specialist interactions focused on gathering key information like, number of villages affected in the district, number of animals affected and data on recent organisation of sandys/fairs and mass movement of animals in the area were recorded. Appropriate biological/ clinical samples like Blood/scab tissues/swabs were collected during the investigation visit and carried samples in cold chain for further laboratory investigations.

Fig 2: Disease investigation undertaken at the farmers doorsteps: Image a- An HF cross heifer with initial signs of high fever and off feed being examined. b,c- Disease investigation team members recording the observations. d- Appearance of clinical signs like generalised nodules on the body in an affected calf. e- An adult Jersey cross cow in advanced stage of recovery leading to slow resorption of nodules/lumps. f-Collection of clinical samples from affected animal for laboratory diagnosis.

PCR based molecular confirmation of Lumpy Skin Disease:

Lumpy skin disease suspected clinical samples were subjected for DNA isolation. The extracted DNA was subjected to PCR amplification with specific primers with an expected amplicon size of 192 bp. The PCR amplified product was resolved in a 2% agarose gel electrophoresis and amplicons were visualized using Gel documentation system. Specific amplification bands were observed corresponding to the expected size of 192 bp in the wells that were loaded with samples, confirming the specific amplification (Figure 3). A no template control (NTC) was used in lane 5 which did not yield any amplification suggesting the amplification is specific.

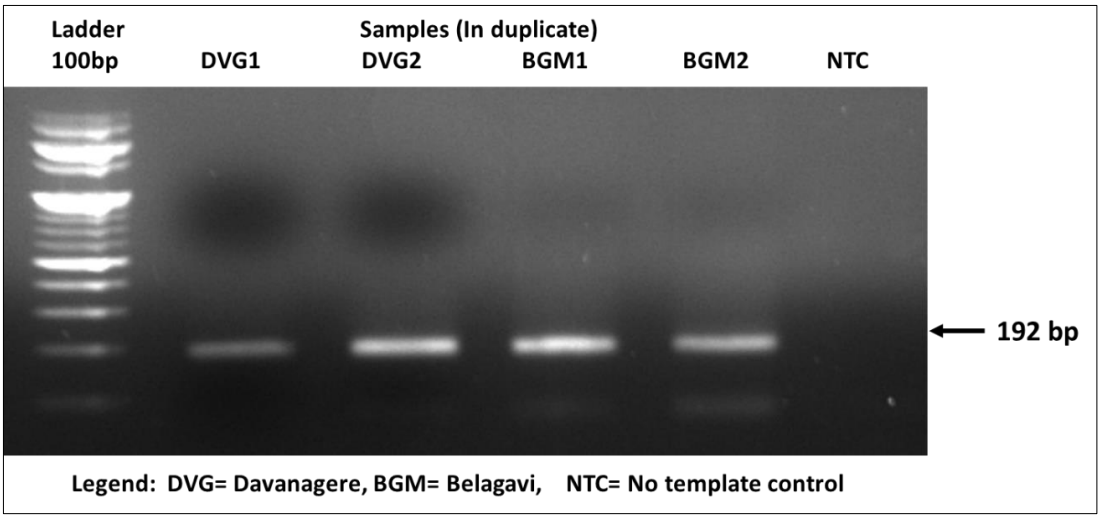


Fig 3: Resolution of PCR products on a 2% agarose gel, showing specific amplification of 192 bp targeting viral attachment gene of LSDV.

Phylogenetic Analysis

The selected PCR product of two positive samples originating from Davanagere and Belagavi were commercially sequenced using sanger sequencing method. The sequence data when BLAST analysed confirmed that the PCR amplicons have exactly matched the lumpy skin disease virus sequences available in the NCBI database, hence confirming LSDV specific amplification. The Phylogenetic tree was then constructed using MEGA X software. Phylogenetic analysis of samples originating from Davanagere revealed about 81.6% sequence identity with LSDV isolates from Zimbabwe (Figure 4). Similarly, Phylogenetic analysis of Belagavi sample showed that it had 91.9% sequence identity with LSDV isolates from Odisha and Bangladesh (Figure 5).

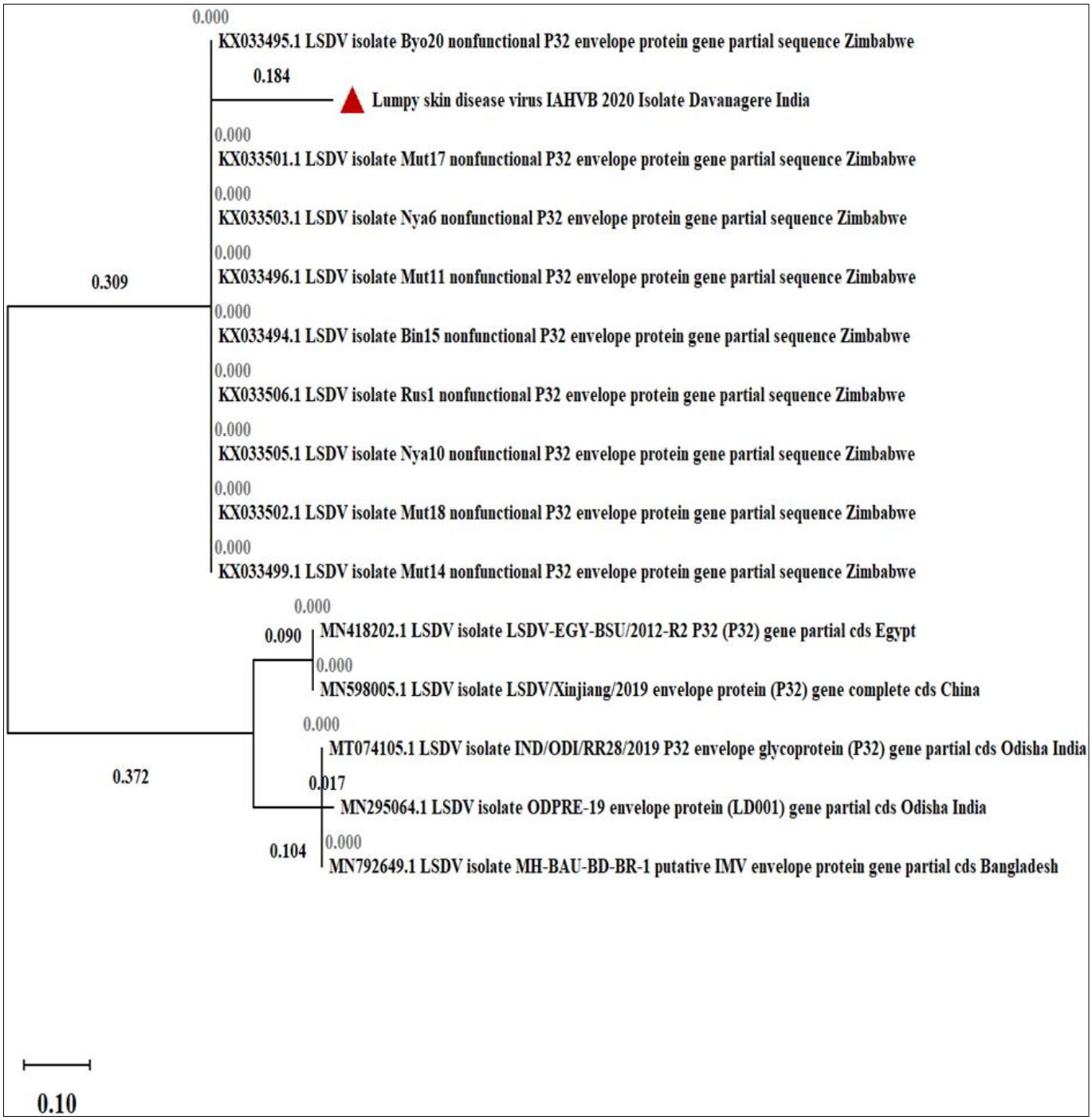


Fig 4: Phylogenetic analysis of Lumpy skin disease virus sequence from Davanagere, India.

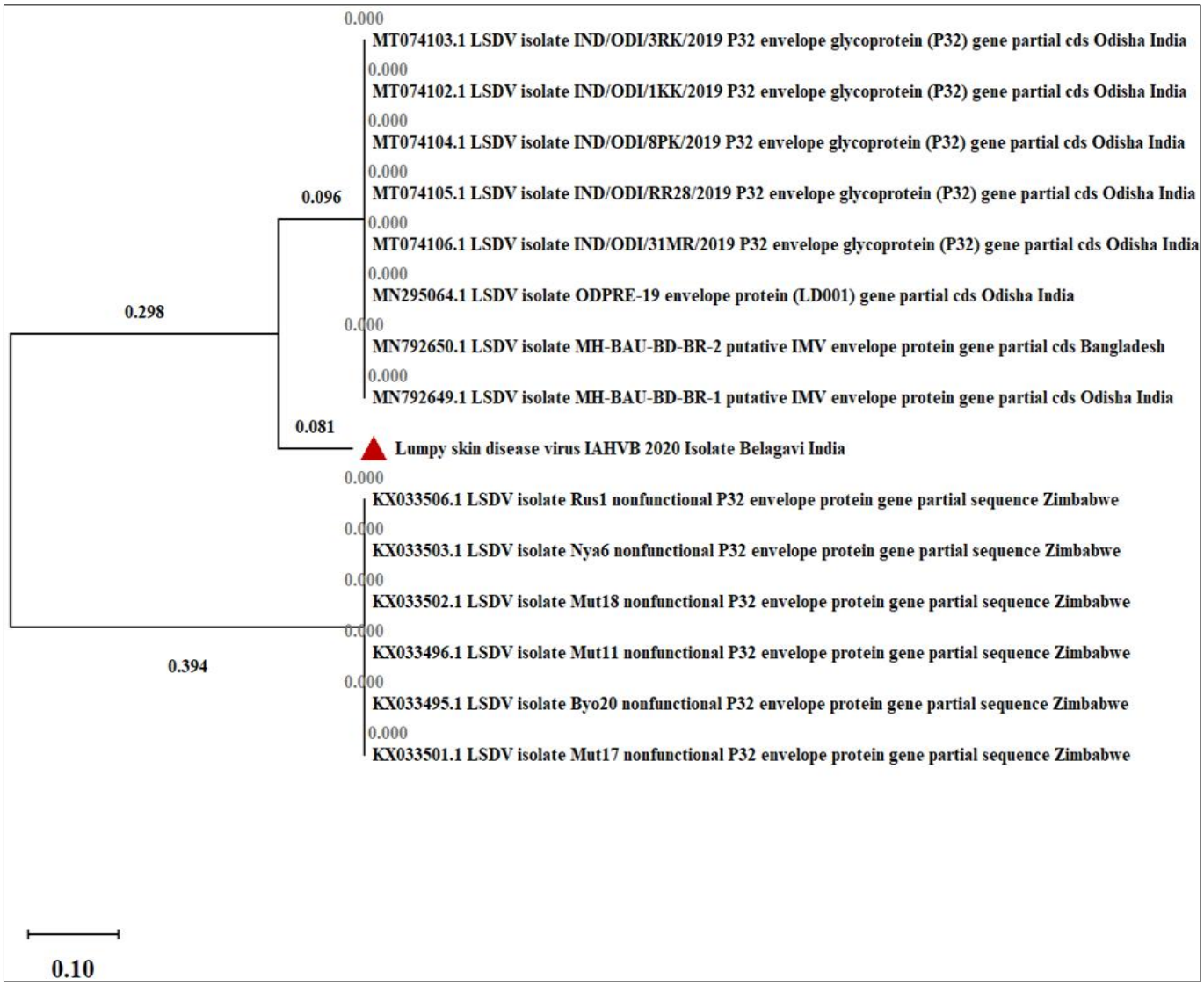


Fig 5: Phylogenetic analysis of Lumpy skin disease virus sequence from Belagavi, India.

Discussion

Lumpy skin disease (LSD), is a highly infectious, notifiable and economically important disease viral disease of cattle. LSD is caused by LSD virus in the genus Capripoxvirus of family Poxviridae. Arthropod vectors play an important role in transmission of LSD virus. LSD is transmitted through direct contact between infected and healthy susceptible animals. The disease is also transmitted by way of indirect contact, mechanical and biological transmission by several arthropod vectors like *Aedes aegypti* mosquitoes, *Culicoides Spps*, and hard Ixodid ticks that are associated with wet, warm summer seasons have been reported. LSDV has traditionally been found in Africa. During the 2012–2018 Eurasian LSD epidemic, the virus expanded its geographical range into the Middle East and Europe. Cattle of all ages and breeds, including wild ruminants, are susceptible to the disease. Lumpy Skin Disease in cattle is characterized by fever, appearance of nodules or lumps on the skin, mucous membranes and internal organs, loss of appetite, debility, enlargement of lymph nodes, skin oedema and sometimes death. However, severity of clinical signs of LSD may be acute or sub-acute form which depends on cattle breeds, age and sex factors. LSD can be diagnosed using appropriate serological and molecular techniques. Lumpy skin disease is considered as a disease of high economic importance because of its ability to compromise food security through loss of draft power, reduced animal production, increased production costs due to increased costs of disease control and disruption in livestock product and by-products trade. Severe economic losses may be incurred due to condemnation of carcass as it damages the hides. Permanent damage to the skin and hides greatly affect leather industry. Attenuated strains of LSD and SPPV are successfully used as vaccines in infected areas. The control of LSD can be achieved through vaccination, restriction of animal movement and eradication of infected and exposed animals. Pathogen environment and host factors are main risk factors for the disease.

Clinical samples collected from ailing animals from investigated outbreaks were processed in the lab for confirmation of the disease. Nucleic acid (DNA) purification was carried out from the Scab tissue/blood samples as per the manufacturers protocol. The extracted DNA was subjected to PCR amplification with specific primers as described by Ireland and Binepal, 1998. The PCR amplification resulted in amplification of a specific DNA fragment with a size of 192 bp, these findings were similar to the observations of previous workers, hence the PCR results of present study co-related with that of the findings of Ireland and Binepal, 1998. The

nucleotide sequence obtained through sequencing of PCR amplicons were BLAST analysed and were aligned with published sequence database in the NCBI GenBank. Sequence analysis revealed that both amplicons matched to the sequences of LSDV, that gives a secondary confirmation that the PCR was specific and amplified part of LSDV viral genome. Phylogenetic tree construction using Neighbor Joining method for relational analysis revealed that the LSDV sample derived from Davanagere district exhibited a sequence homology of 81.6% with LSDV with Asian and African isolates. Similarly, the samples obtained from Belagavi district showed a 91.9% sequence identity with LSDV isolates of Odisha and Bangladesh deposited in GenBank. The findings of the present study are in agreement with the findings of Allam *et al.*, 2020 and Sudhakar *et al.*, 2020 [7] who also showed similar relational phylogenetic studies.

Immunity and Vaccination

Poxviruses have evolved mechanisms of modulating cell death and evading the immune surveillance, thereby targeting both extracellular cytokine signalling as well as intracellular pathways. Host responses that bring about cell death are associated with cell mediated immune clearance of the virus-infected cells and targeting of the cellular enzyme superoxide dismutase (SOD) is an example of the many strategies that poxviruses have evolved to counteract host defences (Douglass *et al*. 2020). Immunity to Capripoxvirus infection is predominantly cell mediated since most progeny viruses remain inside the infected cells. By spreading locally and directly from cell to cell, the virus remains out of the reach of circulating antibodies. The extracellular enveloped Virions, which are released by budding from infected cells, may either infect the neighboring cells or they may escape into the blood and be disseminated throughout the body. A natural resistance to LSDV infection is familiar in cattle, and subclinical LSDV infections are frequent. (Sudhakar *et al*., 2020) [7]. Although LSDV, SPV and GPV have high antigenic similarity and genetic identity (about 95%), genome sequence analyses have shown that these three are phylogenetically distinct. In the vaccinated animals, antibodies will appear 10-15 days post vaccination and reach a peak about 30 days later. A local response to the vaccine generally correlates with good production of antibodies. In case of infection with the virulent wild-type virus, a few animals may pose refractory to LSD vaccination, and fail to develop a local reaction or detectable antibody levels (Krešic *et al*. 2020) [3].

Conclusion

The present study confirmed the ongoing disease outbreak as lumpy skin disease by adopting advanced molecular techniques. Clinical features observed at the field level and the results of the laboratory tests correlated significantly hence validating the sensitivity of diagnostic PCR assay employed in the present study. Further studies may be required to determine the source of Lumpy skin disease virus introduction, modes of transmission and impact on dairy cattle production in Karnataka state. Effective control measures need to be adopted as per the govt of India Advisory on control and containment of lumpy skin disease. Movement restriction in the phase of outbreak and implementation of quarantine measures before induction of new animals into the herd, creating awareness among farmers/stake holders certainly helps to reduce the disease spread and incidence. Preventive prophylactic vaccination of all susceptible animals, along with vector control will help in the successful control of Lumpy skin disease. in the long run.

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