Available online at www.most.gov.bd
Volume 05, Page: 31-36, 2023
DOI: https://doi.org/10.3329/jscitr.v5i1.74003

Ministry of Science and Technology
Government of the People's of Bangladesh
Bangladesh Secretariat, Dhaka

Isolation, Identification and Molecular Characterization of Lumpy Skin Disease Virus for Development of Vaccine

A. A. Bhuyan*, J. Alam, J. Khanom, N. Moonsif, Rubaya, M. A. Alim, M. M. Kamal Hossain, N. Jahan and M. Salimullah

National Institute of Biotechnology, Ganakbari, Ashulia, Savar, Dhaka-1349

Abstract

The highly contagious Lumpy skin disease (LSD) virus has been rapidly spreading across South and East Asia, posing a grave threat to cattle populations in the region. LSD initially emerged in Bangladesh during July 2019, starting in the Chattogram district and rapidly spreading across the entire country. To investigate cases of LSD in cattle, a comprehensive study was conducted in various districts of Bangladesh, including Natore, Kurigram, Dinajpur, Dhaka, and Mymensingh. A total of 58 samples comprising blood, skin scraping, buccal swabs, and nasal swabs were collected for analysis. In order to identify LSDV, the DNA was isolated from the processed samples, and a conentional PCR method was employed utilizing the specific primer designed for the 'P-32 Gene' gene. Out of the blood samples tested, only 29.17 percent yielded a positive result for LSDV, whereas a substantial 80 percent of the skin samples tested positive. This suggests that skin scrapings are a viable specimen for LSD diagnosis using PCR. Five positive samples were partially sequenced utilizing the "ANK Gene" for molecular characterization. Bangladeshi isolates have a strong association with LSDV Kenyan (KSGP 0240, NI-2490) strains and Indian isolates (Ranchi, 2019) strains, based on multiple sequence alignment and phylogenetic analysis. Additionally, the sequencing of all the samples from the five research sites were almost identical, suggesting that a single strain is probably spreading throughout the nation. These findings emphasize the need for continuous monitoring of the genetic makeup and molecular epidemiology of LSDV, which will be useful in the future for the development of vaccines specific to the strains that are then in circulation.

Received:30.04.2023
Revised:02.05.2023
Accepted:10.05.2023

Keywords: Cattle, Chicken embryo, PCR, Sequence, Multiple sequence alignment.

Introduction

Lumpy skin disease (LSD) is a disease of cattle and water buffalo caused by lumpy skin disease virus (LSDV). LSDV is belongs to genus capripoxvirus of subfamily Poxviridae. Other two members of this genus are sheeppox and goatpox virus. Capripoxviruses (CaPVs) cannot be differentiated from one another through serological testing, but they have the ability to provide cross-protection against different strains. The size of the LSDV genome is approximately 151 kilobase pairs (kbp), which includes a central coding region and houses a total of 156 potential genes. Comparison of LSDV with chordopoxviruses of other genera reveals 146 conserved genes. The CaPVs share around 97% sequence identity. The molecular mechanisms underlying the limited host range and virulence of CaPV are still unknown (Tulman et al., 2001, 2002). All age group of animals are

*Corresponding author e-mail: aab_bau@yahoo.com

susceptible to this disease. The disease is characterized by elevated body temperature, numerous nodules in skin, necrotic plaques in the respiratory tract and oral cavity. The intensity of an illness can range from mild to life-threatening. The exact reasons behind why some cattle experience mild symptoms while others experience severe symptoms are currently not understood. LSD was first described from Zambia in 1929, but the virus was isolated in 1940 (Alexander et al., 1957, Babiuk et al., 2008). In subsequent years, the virus gradually expanded its reach across Africa and the Middle East. By 2015, reports of the virus emerged from Greece, the Caucasus, and Russia. The subsequent year saw the continued expansion of its reach towards the east, extending into the Balkans, moving northwards towards Moscow, and stretching westward into Kazakhstan (Agianniotaki et al., 2017, EFSA, 2018). The first occurrence of the disease in Bangladesh was documented at a farm in Karnaphuli Upazila, Chattagram, in July 2019. Subsequently, it rapidly disseminated across the entire country (DLS, 2019). There is currently an ongoing spread of LSD in various regions of the country. The Fisheries and Livestock Ministry has instructed the Livestock Department to swiftly implement necessary actions to avert the outbreak of LSD among cattle. The virus was also reported from India and China in August 2019 (DLS 2019).

LSD is classified as a cross-border disease that impacts animals, with the ability to rapidly spread across international borders and escalate into a widespread epidemic. Consequently, it necessitates collaborative efforts on a regional level to effectively prevent, control, and eliminate the disease. According to current global perspectives, it is widely acknowledged that this disease is rapidly emerging and holds significant consequences. The disease listed by OIE in “List A” due to its rapid spread and severe economic losses. From Bangladesh the disease was reported to OIE on September 2019. Although the mortality is low (about 10%) but it has severe economic consequences. Because it leads prolonged debilitating effects in severely afflicted animals including permanent skin damage, reduced milk production, reduced weight-gain, and sometimes associated with mastitis, orchitis which results in infertility or even sterility in bulls. In approximately 10 percent of gestating cows, the occurrence of infection may lead to abortion. The occurrence of LSD outbreaks can have significant socio-economic effects for small-scale farmers who rely heavily on cattle/buffalo farming for their livelihoods. Furthermore, expenses are incurred from implementing expensive control methods, such as complete or partial eradication of affected animals and subsequent requirements for compensation, farm cleaning, and disinfection. Other costs include medical care for affected animals, vector control, increased laboratory operations (diagnostic tools, reagents, and equipment) as well as heightened expenses for monitoring and surveillance. In areas where cattle are commonly employed for agricultural labor as well as for social and religious events, imposing limitations on these traditional practices can lead to both economic and social upheaval. There is a higher risk of virus contamination in hides compared to meat or milk, and the OIE Terrestrial Animal Health Code has provided specific guidelines for importing hides from countries affected by LSD. LSDV is believed to be primarily transmitted through the bites of insects. Mosquitoes of the Aedes and Culex genera, as well as Ixodid ticks, have been found to carry the virus during certain outbreaks. It is unlikely that direct contact between animals plays a significant role in the spread of the virus. Infection through milk and semen is possible. The transportation of infected cattle can also play a notable role in the dissemination of LSD across extensive geographical areas. The presence of the typical skin nodules is strongly suggestive of LSD and can be confirmed by detecting virus DNA or antibodies (Lamien et al., 2011, Agianniotaki et al., 2017, Menasherow et al., 2014; Vidanovic et al., 2016). The risk factor of LSDV in Bangladesh is still hazy and no study have been done yet on this. So molecular characterization of LSDV is very necessary for this time in Bangladesh.

Materials and Methods

Study area and samples collection

A total of 48 blood samples were collected from Dinajpur and Kurigram in 2020 and 10 samples (blood-10, skin nodules-10, and Buccal-10 and nasal swabs-10) from Natore and Dhaka (Dhamrai) in 2021. Samples were collected from cattle

showing clinical signs suspected to be LSD. The blood samples were collected in tubes containing anticoagulants. Skin nodules were obtained by washing the infected area with clean water and then removing hairs with a sterilized scalpel blade. Buccal and nasal swabs were collected aseptically into swab collection tubes containing MEM. The samples were then transported in double-walled iceboxes to the Animal Biotechnology division of the National Institute of Biotechnology (NIB). All the samples were stored at -20 º C until further analysis.

Sample Processing

For skin samples, a thin layer of skin was cut in a sterile manner using a sterile blade and chopped into small pieces. After weighing they were macerated using mortar and pestle and a suspension (10% w/v) was made with PBS-pH 7.4 containing antibiotic and antimycotic solution. Then the tissue homogenates were centrifuged at 1000 rpm for 7 minutes and the supernatant was collected in a sterile aliquot for LSDV isolation and identification.

Viral DNA Extraction

DNA was extracted from blood (Ghatak et al., 2013), skin samples (Markoulatos et al., 2000), swabs (Ghatak et al., 2013), and infected CAM (Sambrook et al., 1989) and stored at -20 °C until used in PCR.

Molecular identification

A conventional PCR was applied for the initial detection of the LSDV using capripoxvirus-specific primers (Sharawi and Abd El-Rahim, 2011) targeting partial P-32 gene (Table 1).

PCR reaction was performed on a 12.5 µl reaction scale. The reaction consisted of 6.25 µl of 2x master mix (2x Taq Master-Mix (Dye), CWBIO), 1µl sample (~ 100 ngDNA), 0.5µl forward primer (20 pmol), 0.5µl reverse primer (20 pmol) and 4.25 µl molecular grade nuclease-free water. The amplification was conducted in a thermal cycler (Biometra Tone-Analytik Jena, Germany) applying the following conditions: initial denaturation at 95°C for 5 mins, 40 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1 min and a final extension period is 72°C for 5 min. DNA from commercially available LSDV vaccine (Lympyvax®) was used as a positive control. PCR products were run in 1.5% and visualized under a UV transilluminator.

Amplification and nucleotide sequencing for molecular characterization

The PCR products were separated by gel electrophoresis (1.5% gel) and then purified using a PCR purification kit (Purelink^{TM}, Thermo Fisher Scientific) according to manufacturer instructions. Sequencing was performed using an automated DNA sequencer 3110 Genetic Analyzer (Applied Biosystems, USA) and edited with SeqEscape V2.7 software. ANK gene was amplified employing primer pairs according to Kumar et al., 2021 in a 50 µl volume.

Phylogenetic Analysis

The ANK gene sequences were edited to 636 bp respectively using BioEdit software version 7.2.5 and they were used for phylogenetic analysis. The reference sequences for other LSDVs, GTPVs, and SPPVs used in this study were retrieved from the GenBank database. For comparative analysis, multiple sequence alignment was done using the MUSCLE algorithm in MEGA 11. The neighbor-joining method was used along with the Tamura 3-parameter method to construct the phylogenetic trees and bootstrap analysis was used with 1,000 replicates to estimate the reliability of phylogenetic relationships.

Results and Discussion

Outbreak investigation and clinical signs

The most common clinical signs observed in the LSD-suspected animals from all four districts (Kurigram, Dinajpur, Natore, Dhaka) were firm nodules of various sizes on the skin of the neck, head, perineum, udder, teats, and sometimes throughout the whole body; fever; loss of appetite; depression; nasal and ocular discharge, etc. In some of the infected cattle, the nodules were seen as ulcerated and sometimes indurated. Nasal discharge was present in only a few (10/58) animals. According to OIE, on 22nd July 2019 from among

360 susceptible cattle presenting external clinical signs of LSD, 66 (18.33%) were identified as affected by LSDV. Later performing real-time PCR, a true scenario of LSD outbreak had been revealed by Central Disease Investigation Laboratory (CDIL). The recent and unprecedented spread of LSDV in Bangladesh and several other countries underscores the critical need for increased research into this rapidly emerging pathogen.

Molecular detection of LSDV

About 172 bp product from the P-32 gene of capripoxvirus was amplified and a representative image is shown in Figure 1. The expected amplicon size that was detected in the clinical samples of 22 cattle out of 58, which was around 37.93 % of the total number of cattle. The amplified fragments were further confirmed by sequencing. The findings were consistent with prior studies that confirmed the feasibility of utilizing PCR for detecting LSDV in various types of samples, including biopsy samples, tissue culture, skin, blood, and semen (Awad et al., 2010). This Skin nodules and scabs provided more obvious bands (80 %) than blood samples (29.17%) indicating their high virus concentration. The outcomes were consistent with expectations in agreement with Carn & Kitching, 1995, who stated that the viral concentration in the nodules is significantly higher than the virus that is present in the bloodstream during viremia. As a result, skin nodule can be regarded as more suitable sample for LSDV detection. Additionally, scabs are the greatest sample material because they are simple to collect and do not require local anesthesia, and withstand long transport in different temperatures. The buccal and nasal swab used in this study did not show any positive result though skin scabs from the same animals showed positive bands. It might be possible that virus concentration was not enough in the swab samples. More studies including swab samples are required for confirmation.

Sequencing of ANK and Phylogenetic Analysis

For each corresponding gene, every sequence from the four study regions showed 100% identity among each other and they clustered together in their respective phylogenetic trees. Moreover, all the sequences of capripoxviruses obtained from GenBank could be classified into three distinct clusters of LSDV, GTPV and SPPV (Figure 2). Phylogenetic analysis revealed that despite having highly conserved regions these three species were classified into three distinct clusters of LSDV, GTPV, and SPPV indicating they have significant genetic differences among themselves (Tulman et al., 2002).

Analysis of ANK Gene

In the analysis of Ankyrin Repeat, the nucleotide sequence homology between LSDV isolates from this present study and LSDV field strains from other countries were in the range of 98.59% to 100% and the highest homology was found with Kenyan strains and one Indian Isolate. As enough isolates from India were not present in the database, the comparison couldn't be made properly. Amino acid sequence alignment of this gene showed several sequence differences at different positions. Among them, at position 71 asparagine (N71) was found in all LSDVs from Bangladesh, Kenya, India, and one Russian and Kazakhstan isolate whereas lysine (K71) was found instead in all other analyzed field-strains from Africa, the Middle East, and Europe (Figure 3). In the Phylogenetic tree analysis of Ankyrin repeat, isolates from the present study were also tightly clustered with Neethling 2490 and KSGP 0240 Kenyan strains and one Indian isolate available in the database. Other isolates from Europe, The Middle East, and Africa showed slight genetic differences forming another clade though they shared the same node with the isolates from this study and also were present in the same subgroup I. Kenyan LSDV strains such as LSDV KSGP 0240, LSDV NI2490 (1958), and LSDV Kenya (1950, but not sequenced until recently) caused LSD outbreaks in Kenya in the past (Tulman et al., 2001).

Conclusion

Lumpy Skin Disease was recognized as the primary cattle health issue, resulting in significant economic loss due to decreased weight gain, permanent damage to hides, loss of milk production, a prolonged debilitating clinical course, temporary or permanent infertility, or even sterility in bulls, and abortion of pregnant cows. In the study area, Farmers and dairy farm owners were well aware of

LSD, as it caused a moderate increase in morbidity and mortality, which may have resulted in direct and indirect economic losses.

PCR can be used for the efficient and rapid diagnosis of this disease and also it doesn't require any specific reagents that cannot be obtained commercially. In this study, Skin samples showed a higher percentage of positive results than blood samples indicating a higher concentration of this virus in the skin nodule rather than in blood. So, skin scabs may be used for the identification of this disease which is also easy to collect.

Again, this study also confirmed that the LSDV strains causing 2019 outbreaks in Bangladesh and India are very similar, suggesting a common exotic source of LSDV introduction. They are also very similar to the historical LSDV KSGP 0240-like field strains from Kenya but are different from contemporary field strains circulating in Africa, the Middle East, Central Asia, and Europe and some Vaccines strains from South Africa. It may be possible that this disease entered Bangladesh from India but how such disease was encountered in India is still unknown, most probably by the import of animals or animal products from Africa.

Acknowledgements

The authors like to kindly acknowledge the Ministry of Science and Technology, Government of the People's Republic of Bangladesh for providing their financial support through providing the Special Allocation Funds 2,50,000 BDT approving the project (ID No. BS-325, year 2021-2022) entitled "Isolation, identification & molecular characterization of lumpy skin disease virus for development of vaccine".

Conflict of Interests

The authors have no disclosure to make that qualifies as a conflict of interest.

Statement of Author's Credit

Conceptualization: A. A. Bhuyan, J. Alam; Methodology: A. A. Bhuyan, J. Khanom, N. Moonsif, Rubaya; Validation: A. A. Bhuyan, J. Khanom, M. M. Kamal Hossain' Formal analysis: A. A. Bhuyan, J. Alam; Investigation: A. A. Bhuyan, J. Alam, J. Khanom, N. Moonsif, Rubaya, M. A. Alim; Resources: A. A. Bhuyan, M. A. Alim, N. Jahan and M. Salimullah; Data Curation: A. A. Bhuyan, J. Alam, J. Khanom; Writing-Review & Editing: A. A. Bhuyan, J. Alam, J. Khanom; Supervision: J. Alam and M. Salimullah; Project administration: A. A. Bhuyan and N. Moonsif; Funding acquisition: Ministry of Science and Technology and National Institute of Biotechnology.

References

Abutarbush, S. M., Hananeh, W. M., Ramadan, W., Al Sheyab, O. M., Alnajjar, A. R., Al Zoubi, I. G., Knowles, N. J., Bachanek-Bankowska, K., & Tuppurainen, E. S. M. (2016). Adverse reactions to field vaccination against lumpy skin disease in Jordan. Transboundary and Emerging Diseases, 63(2), e213–e219.

Agianniotaki, E. I., Tasioudi, K. E., Chaintoutis, S. C., Iliadou, P., Mangana-Vougiouka, O., Kirtzalidou, A., Alexandropoulos, T., Sachpatzidis, A., Plevraki, E., & Dovas, C. I. (2017). Lumpy skin disease outbreaks in Greece during 2015–16, implementation of emergency immunization and genetic differentiation between field isolates and vaccine virus strains. Veterinary Microbiology, 201, 78–84.

Alexander, R.A., Plowright, W. & Haig, D.A., 1957. Cytopathogenic agents associated with lumpy skin disease of cattle. Bulletin of Epizootic Diseases of Africa, 5, 489–492.

Babiuk, S., Wallace, D. B., Smith, S. J., Bowden, T. R., Dalman, B., Parkyn, G., Copps, J., & Boyle, D. B. (2009). Detection of antibodies against capripoxviruses using an inactivated sheeppox virus ELISA. Transboundary and Emerging Diseases, 56(4), 132–141.

Department of Livestock Services (DLS) 2019. Situation Report: Lumpy Skin Disease in Bangladesh. Paper presented on Seminar entitled "Lumpy skin diseases in Bangladesh: Status, Challenges and Way Forward" organized by Livestock Division of BARC on 19 December 2019.

European Food Safety Authority (EFSA). 2018. Scientific report on lumpy skin disease II. Data collection and analysis. European Food Safety Authority Journal, 16(2), 5176, 33.

Ghatak, S., Bose Muthukumaran, R., & Kumar Nachimuthu, S. (2013). A Simple Method of Genomic DNA Extraction from Human Samples for PCR-RFLP Analysis. https://doi.org/10.7171/jbt.13-2404-001

Green, H.F., 1959. Lumpy skin disease: Its effect on hides and leather and a comparison in this respect with some other skin diseases. Bulletin of Epizootic Diseases of Africa, 7, 63.

Kumar, N., Chander, Y., Kumar, R., Khandelwal, N., Riyesh, T., Chaudhary, K., Shanmugasundaram, K., Kumar, S., Kumar, A., Gupta, M. K., Pal, Y., Barua, S., & Tripathi, B. N. (2021). Isolation and characterization of lumpy skin disease virus from cattle in India. PLoS ONE, 16(1 January), 1–13. https://doi.org/10.1371/journal.pone.024102

Lamien, C.E., Goff, LE, Silber, C.,, Wallace, R., Gulyaz, D.B., Tuppurainen, V., Madani, E., Caufour, H., Adam, P., El-Harrak, T., Luckins, M., Albina, A.G., & Diallo, A., 2011. Use of the Capripoxvirus homologue of Vaccinia virus 30 kDa RNA polymerase subunit (RPO30) gene as a novel diagnostic and genotyping target: Development of a classical PCR method to differentiate goat poxvirus from sheep poxvirus. Veterinary Microbiology, 149, 30-39.

Le Goff, C., Lamien, C.E., Fakhfakh, E., Chadeyras, A., Aba-Adulugba, E., Libeau, G., Tuppurainen, E., Wallace, D.B., Adam, T., Silber, R., 2009. Capripoxvirus G-protein-coupled chemokine receptor: a host-range gene suitable for virus animal origin discrimination. Journal of general virology 90, 1967-1977.

Menasherow, S., Rubinstein-Giuni, M., Kovtunenko, A., Eyngor, Y., Fridgut, O., Rotenberg, D., Khinich, Y. & Stram, Y., 2014. Development of an assay to differentiate between virulent and vaccine strains of lumpy skin disease virus (LSDV). Journal of Virological Methods, 199, 95-101.

OIE (2017) Lumpy skin Disease OIE Terrestrial Mannual 2017 Chapter 2.4.13. (n.d.).

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). Molecular cloning: a laboratory manual. (Issue Ed. 2). Cold spring harbor laboratory press.

Tulman, E. R., Afonso, C. L., Lu, Z., Zsak, L., Sur, J.-H., Sandybaev, N. T., Kerembekova, U. Z., Zaitsev, V. L., Kutish, G. F., & Rock, D. L. (2002). The genomes of sheeppox and goatpox viruses. Journal of Virology, 76(12), 6054–6061.

Vidanovic, D., Sekler, M., Petrovic, T., Debeljak, Z., Vaskovic, N., Matovic, K. & Hoffmann, B., 2016. Real-time PCR assays for the specific detection of field Balkan strains of lumpy skin disease virus. Acta Veterinaria-Beograd, 66, 444–454.

Yeruham, I., Perl, S., Nyska, A., Abraham, A., Davidson, M., Haymovitch, M., Zamir, O., & Grinstein, H. (1994). Adverse reactions in cattle to a capripox vaccine. The Veterinary Record, 135(14), 330–332.