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In-silico exploration of species-specific early gene putative promoter motif of the Indian lumpy skin disease virus vaccine strain

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Abstract
This study focuses on the identification of conserved sequences within the early gene promoter region of the Lumpy Skin Disease Virus (LSDV) genome. These sequences are located upstream of the transcription initiation sites of specific genes. Genomic data for both LSDV and Vaccinia virus (VACV) were obtained from the NCBI nucleotide (nt) database in FASTA and GB file formats. Using the Genome Annotation Transfer Utility (GATU) online software, LSDV gene sequences were annotated by comparison with the VACV genome. These genes were categorized as "Early" or "Late" based on published poxvirus literature. Specifically, early genes of LSDV were selected to identify consensus and conserved sequences in their promoter regions. Spacer regions between these conserved sequences and the gene's start codon were analyzed. A 103nt segment upstream of the translation initiation site from 34 early genes was retrieved from the NCBI database in FASTA format. The Multiple Em for Motif Elicitation (MEME) Suite 5.5.4 online program was employed to identify conserved sequences (motifs) within the early promoter region of LSDV. The results revealed a 15nt consensus sequence (AAAAATGAAAAAAAA) at the core of the 34 numbers of early gene promoter motifs for LSDV genes. Importantly, variations in the spacer lengths between the coding region and the regulatory early promoter motif were observed among early genes, potentially influencing gene expression.

Keywords: Lumpy skin disease virus (LSDV), promoter motifs, *in-silico* analysis, cattle disease, capripoxvirus

Introduction
Lumpy skin disease (LSD) is a highly contagious and economically significant viral infection that affects cattle. The causative agent, Lumpy Skin Disease Virus (LSDV), belongs to the Capripoxvirus genus within the Poxviridae family (Gupta *et al.*, 2020) [4]. LSDV infection in cattle leads to a range of clinical manifestations, including fever, generalized skin nodules, and mucosal lesions, ultimately resulting in severe economic losses for the livestock industry (Kumar and Tripathi, 2022) [9]. In recent years, there has been a notable geographical expansion of LSD outbreaks. Historically, LSD was confined to African countries since its initial report in Zambia in 1929 (Tuppurainen *et al.*, 2012) [15]. However, a pivotal shift occurred in 2012 when it began spreading into various European nations. In 2019, the presence of LSD was officially documented in Asia, with cases reported in North West China, Bangladesh, and India, marking the virus's entry into the continent (Kumar *et al.*, 2023; Kumar *et al.*, 2021) [7, 8]. Subsequently, by 2020, the disease had proliferated across multiple Asian countries, emphasizing the urgent need for extensive research and tailored control strategies to protect the diverse livestock populations of India.
One promising strategy in the fight against LSDV is the use of vaccine strains specifically designed to confer immunity against the virus. In particular, the "Lumpi-ProVacInd" live-attenuated LSD vaccine, developed using the vaccine strain LSDV/RCH/P50, originating from India, has shown significant promise (Kumar *et al.*, 2023) [7]. This vaccine strain has proven effective in cell culture, laboratory animal models, and natural host cattle, underscoring its potential as a valuable tool in mitigating the impact of LSD in endemic regions and curbing the disease's spread (Kumar *et al.*, 2023) [7].
Beyond its utility against LSD, there is a growing interest in LSDV vectored vaccines as a versatile platform for combating various cattle diseases caused by different pathogens.

This approach leverages the unique characteristics of the poxvirus vector system, allowing for the expression of recombinant genes. Poxviruses, capable of incorporating up to 25 kilobases of heterologous genes, offer a powerful platform for creating recombinant vaccines that can target a wide spectrum of infectious diseases (Smith and Moss 1983) [12]. The adaptability of poxvirus vectors has extended their application to development of recombinant vaccines for human pathogens like Herpes virus, Hepatitis B virus, Influenza virus, and HIV, as well as a range of animal diseases pathogens such as Rabies, Rinderpest virus, Bluetongue virus, FMD virus, PPRV, CSFV and PRRSV (Sánchez-Sampedro *et al.*, 2015; Jiménez-Cabello *et al.*, 2020) [11, 6]. Furthermore, poxvirus vectors have been explored for therapeutic cancer vaccines, harnessing the immune system's potential to combat cancer cells (Hodge *et al.*, 1995; Mulryan *et al.*, 2002; Sánchez-Sampedro *et al.*, 2015) [5, 10, 11]. The enduring immunity and safety profile of these vaccines make them valuable tools for immunization against diseases.

Promoter sequences, essential for gene expression regulation, play a crucial role in optimizing recombinant poxvirus-based vaccines. In the case of VACV, gene expression is specifically controlled through early, intermediate, and late promoters (Yang *et al.*, 2011; Yang *et al.*, 2013) [17, 18]. The precise identification of suitable promoters is essential for achieving optimal expression of recombinant genes within the LSDV genome. This is a critical aspect of improving the efficacy of recombinant vaccines since the efficiency of gene expression directly impacts the protective immune response generated by the vaccine. This study focuses on the *in-silico* identification of putative promoter motifs for early genes of the Indian Lumpy Skin Disease Virus vaccine strain (LSDV/RCH/P50). Prior research has differentiated and annotated the early, intermediate, and late genes of the lumpy skin disease virus (Tulman *et al.*, 2001) [14]. Thus, we consider these previously identified genome sequences as references for our current study to identify specific regions in the genome sequences under investigation.

Materials and Methods

Selection of Reference Genomes: In the present study, two reference genomes of LSDV were selected. The first reference genome, Accession no. AF325528, represents the standard LSDV genome. The second reference genome, Accession no. OK422494, pertains to the LSDV vaccine strain-LSDV/RCH/P50 genome used in the current investigation.

Determination of Transcription Start Sites and Promoter Sequences: To identify the transcription start sites and promoter sequences of the early genes in the LSDV genome, firstly the complete genome sequences of LSDV, specifically Accession nos. AF325528 and OK422494, were obtained in both GenBank (GB) file and FASTA file formats from the NCBI Genome Browser (https://www.ncbi.nlm.nih.gov/gene). The early genes were initially identified in the reference genome AF325528 and the reference genome of Vaccinia virus (Accession no. M35027.1). The open reading frames (ORFs) within the complete LSDV genome were compared using Genome Annotation Transfer Utility (GATU, https://4virology.net/virology-ca-tools/gatu/) and annotated against the reference genome of VACV (M35027.1) (Tulman *et al.*, 2001) [14]. Simultaneously, Specific early genes in the LSDV genome (Accession no. OK422494) were identified using SnapGene Viewer (Version 7.0.3). Genes that initiated with the ATG codon (start codon) were selected. Sequences encompassing the 100 nt upstream of the coding sequences were collected as viral gene promoter for further analysis.

Promoter Sequence Retrieval: The promoter sequences that 100 nt upstream of TSS including the initiation codon (ATG), for each of the early genes were retrieved in FASTA format. This format was chosen as it is compatible with in-silico analysis in MEME online software (Timothy *et al.*, 2015) [13]. The initiation codon (ATG) was included in each of the promoter sequences, given the tendency of poxvirus promoters to overlap with the open reading frame, as previously mentioned.

Promoter motif analysis of LSDV: We employed the MEME suite (version 5.5.4) to identify putative promoter motifs for early gene of the LSDV. Initially, early gene promoter sequences were retrieved and organized in FASTA format. Subsequently, the MEME software was utilized to analyze these promoters and identify potential motifs. To refine the results, we set a limit of up to three motifs and constrained the width of the discovered binding motifs to a range between 4 and 20 base pairs, aligning with established Position Weight Matrices (PWMs). This analysis unveiled significant promoter motifs in the given sequences. Additionally, we meticulously examined the spacer regions located between the conserved sequences, extending up to the transcription start codon of the genes. This approach provided a systematic and comprehensive understanding of putative promoter motifs, contributing to the comprehension of gene expression of early genes of LSDV.

Results

Identification of LSDV Early Genes and promoter in the Vaccine Strain: The present study commenced with the selection of the reference genome sequence AF325528 for LSDV, which was previously known to contained 41 early genes. Subsequent analysis revealed the successful identification of 34 early genes within the genome of the LSDV vaccine strain AF325528 as compared to reference genome (Table 1). These findings highlight the genetic variation between the LSDV reference genome and the LSDV vaccine strain. The gene identification process served as precursor for the subsequent promoter identification. Accordingly, the upstream 100 nucleotides of each of the 34 early genes in the vaccine strain were retrieved in the 5' to 3' direction from NCBI (Table 1).

Identified promoter motifs of LSDV early gene: The present study focused on the identification of putative promoter motifs specific to the early genes of the LSDV vaccine strain, LSDV/RCH/P50. Using the MEME suite online software, we conducted an in-depth analysis of the promoter regions associated with these genes (Figure 1A). Our analysis revealed a highly conserved 15 nt motif, 'AAAATGAAAAAAAA,' which was consistently found in all 34 (100%) of the LSDV early gene promoters (Figure 1B). The identified promoter regions exhibited a significant enrichment in Adenine-rich motifs, indicating a distinct pattern associated with LSDV early genes. Additionally, our analysis identified two other motifs in a subset of the LSDV early gene promoters. A 15 nt conserved sequence, 'YAGCWCTAWWBKYGS,' which was present in 6 out of the 34 promoter sites and an 8 nt conserved sequence, 'GTKGGWGT,' which was also identified in 6 out of the 34 LSDV promoter sites.

Table 1: Comprehensive overview of the genomes and putative early promoters of Lumpy Skin Disease virus strains employed in this study

Reference LSDV Genome (Accession no. AF325528)		LSDV genome used in present study (Accession no. OK422494)		Protein name or function
ORF	ORF Position	Corresponding ORF	Position of Promoter (100 bp upstream of ATG+ initiation codon)	
LSDV009	6389–5700	LSDVP50_00008	c(5909-6011)	A-amanitin-sensitive protein. A52R-like protein
LSDV010	6929–6444	LSDVP50_00009	c(6449-6551)	LAP/PHD-finger protein
LSDV011	8118–6976	LSDVP50_00010	c(7638-7740)	G protein-coupled CC chemokine receptor
LSDV012	8860–8228	LSDVP50_00011	c(8379-8481)	Ankyrin repeat protein
LSDV013	9924–8902	LSDVP50_00012	c(9443-9545)	IL-1 receptor
LSDV017	11552–11025	LSDVP50_00016	c(11071-11173)	Integral membrane protein, apoptosis regulator
LSDV018	12034–11597	DUT	c(11553-11655)	dUTPase
LSDV020	14820–13858	F4L	c(14338-14440)	Ribonucleotide reductase, small subunit
LSDV021	15121–14864	LSDVP50_00021	c(14639-14741)	Hypothetical protein
LSDV022	15500–15165	LSDVP50_00022	c(15018-15120)	Hypothetical protein
LSDV023	15949–15734	LSDVP50_00023	c(15467-15569)	Hypothetical protein
LSDV029	22624–22190	LSDVP50_00030	c(22142-22244)	Hypothetical protein
LSDV030	23360–22704	LSDVP50_00031	c(22878-22980)	Hypothetical protein
LSDV032	25176–23755	PAPL	c(24695-24797)	Poly(A) polymerase (PAPL)
LSDV034	27925–27395	E3L	c(27444-27546)	PKR inhibitor, host range
LSDV036	28591–27989	LSDVP50_00036	c(28110-28212)	RNA polymerase subunit (RPO30)
LSDV045	40200–39373	I3L	c(39720-39822)	DNA-binding phosphoprotein
LSDV055	49453–49641	RPO7	48875-48977	RNA polymerase subunit (RPO7)
LSDV061	53928–54203	LSDVP50_00064	53351-53453	Protein L2
LSDV067	57402–57995	LSDVP50_00070	56825-56927	Host range protein
LSDV071	60022–63876	LSDVP50_00073	59445-59547	RNA polymerase subunit (RPO147)
LSDV076	68522–69190	LSDVP50_00078	67945-68047	Late transcription factor (VLTF-4)
LSDV079	70682–73207	LSDVP50_00081	70105-70207	mRNA capping enzyme, large subunit
LSDV086	79895–80533	D9R	79318-79420	mut T motif
LSDV089	84100–83240	LSDVP50_00091	c(83621-83723)	mRNA capping enzyme, small subunit; (VITF)
LSDV099	93723–94592	VITF3S	93097-93199	Intermediate transcription factor (VITF-3)
LSDV107	100199–99915	LSDVP50_00108	c(99674-99776)	Core protein A15
LSDV112	103931–105220	LSDVP50_00114	103305-103407	DNA polymerase processivity factor
LSDV115	105723–106877	VITF3L	105097-105199	Intermediate transcription factor (VITF-3)
LSDV116	106911–110378	LSDVP50_00117	106285-106387	RNA polymerase subunit (RPO132)
LSDV119	112173–111268	LSDVP50_00120	c(111645-111747)	RNA polymerase subunit (RPO35)
LSDV125	115216–116079	LSDVP50_00127	114590-114692	Hypothetical protein
LSDV136	129453–129911	LSDVP50_00137	128827-128929	A52R-like family protein
LSDV137	129980–130984	LSDVP50_00138	123354-123456	Hypothetical protein

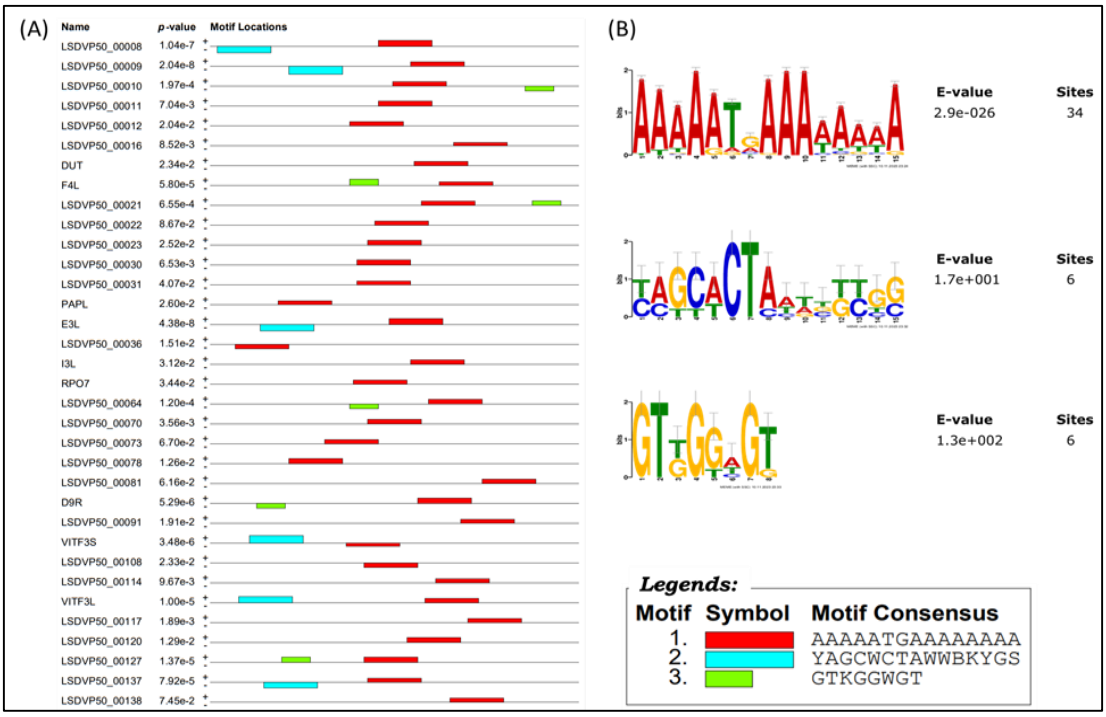

Fig 2: Putative promoter motif for early genes of the Lumpy Skin Disease virus as identified through MEME suite analysis tools. In (A) Identified motifs in each promoter sequence along with their respective significance levels (p-values). (B) Displays three distinct motif nucleotide types, along with their E-values, indicating the statistical significance of the motifs, and the specific targeted sites

Spacer analysis between promoter motif and TSS

The mostly found motifs were predominantly located within the middle of the promoter region, suggesting a conserved pattern in their positioning (Table 2). The analysis revealed that the minimum distance from the start point of the putative promoter motif to the Translation Start Site (TSS) was 20 nt in the early gene PAPL. In this case, the gap between the putative promoter motif and the TSS was 66 nt, indicating a substantial distance between the motif and the translation initiation codon. Conversely, the maximum distance of the start point of the putative promoter motif was observed at 77 nt in the early gene LSDVP50_00081, also on the positive strand. In this instance, the gap between the putative promoter motif and the TSS was notably shorter, measuring only 9 nt. Collectively, our findings suggest that the early gene putative promoter motifs of LSDV are located in a range of 9 to 66 nt from the translation initiation codon. This variability in the distance between the motif and the TSS may play a role in regulating gene expression and could have implications for the efficiency and specificity of recombinant vaccine expression using LSDV as a vector.

Table 2: Putative promoter motif identification via *in-silico* analysis for the Lumpy skin disease virus vaccine strain LSDV/RCH/P50 (Accession no. OK422494)

ORF name	Strand	Start (Gap*)	*p*-value	Targeted sites of promoter motif within 103 nucleotide position		
E3L	+	51 (35)	2.74E-07	CATAAACTAT	AAAAATGAAAAAAAA	AAATTTTTTA
LSDVP50_00008	+	48 (38)	2.74E-07	GAATATATAT	AAAAATGAAAAAAAA	TATTACACTT
D9R	+	59 (27)	1.65E-06	CCATCTAATT	AAAAATGAAAAATAA	TAACGTTTAT
LSDVP50_00127	+	44 (42)	1.79E-06	TGTGGATATA	AAAAATGAAATAGAA	TATTAATTAT
LSDVP50_00078	+	23 (63)	2.54E-06	AATTTTTTAT	AAAAATGAAATAATA	AAGACTTGTT
LSDVP50_00030	+	42 (44)	2.54E-06	GTATGCGATC	AAAAATGAAAAAGCA	TTCGTTTTTT
LSDVP50_00120	+	56 (30)	3.91E-06	TCATTAAATA	AAAAGTCAAAAAAAA	ATTTTAAAAG
LSDVP50_00011	+	48 (38)	3.91E-06	AACACTTTAC	AAAAATGAAATATAA	CTTTTTATTA
LSDVP50_00064	+	62 (24)	6.42E-06	ATAGAAAAAT	AAAAGTGAAAAAAGA	AAAGAAAAAA
LSDVP50_00023	+	45 (41)	6.98E-06	AAAAATCATT	AAAAATGAAACATAA	ATCATAGCTA
LSDVP50_00070	+	45 (41)	9.80E-06	ATTAATGAAA	AAAAAGCAAAAAAAA	GTGATCTATT
LSDVP50_00108	-	44 (-)	1.37E-05	TATATTACCA	AAAAATAAAATAAAA	CAGCAAGACA
I3L	+	57 (29)	1.37E-05	TTTAAATAAG	AAAAATGAAATATTA	TCAAATTGAG
LSDVP50_00010	+	52 (34)	1.37E-05	ATTAAAAAAA	AAAAATAAAATAAAA	GTGTATGATT
VITF3L	+	61 (25)	1.60E-05	TTCCGTATAA	AAAAATAAAAAAATA	GTTAAAAAAC
LSDVP50_00009	+	57 (29)	1.60E-05	GCATTGGATA	AAAAATAAAAAAATA	ATAACAACAA
LSDVP50_00081	+	77 (9)	1.76E-05	TTAAGTGAAA	AAAAATAAAACAAAA	AATAAAATAA
LSDVP50_00036	-	8 (-)	1.94E-05	CCATTAATGC	AAAAATGGAAAATAA	TACACCA
LSDVP50_00073	+	33 (53)	2.64E-05	ATAAAAGAAA	AAAAAAGAAAAAAAG	AAAAAATCAG
LSDVP50_00021	+	60 (26)	2.64E-05	TATTTCTATA	AAAAATAAAAAAAAG	TATAAATTTT
LSDVP50_00012	+	40 (46)	2.64E-05	AATAACAACT	AAAAATTAAAAAAAA	AATATTTTTT
LSDVP50_00114	+	64 (22)	2.82E-05	TATTAAAAAT	AAGAATGAAATAACA	TAAAATTAGA
LSDVP50_00022	+	47 (39)	3.05E-05	ATTTAAGGTT	AAAAAACAAAAAAAA	TAAACTAAGT
DUT	+	58 (28)	3.05E-05	TTTTTAAAAA	AAAAAACAAAAAAAA	AATATCCTAA
LSDVP50_00117	+	73 (13)	3.56E-05	TTAATTAATT	TAAAGTGAAAAAAAA	CACTATTTTG
RPO7	+	41 (45)	3.72E-05	GTTTTATTTT	AACAATGAAAACATA	ATTAGAAATA
LSDVP50_00138	+	68 (18)	4.13E-05	GTTAAAAAAT	AAAAATGAAAATGTA	CTTTTGAATA
LSDVP50_00137	+	45 (41)	4.13E-05	TTTAAACTTA	AAAAGTTAAAAAAAA	ATAAAATTTT
LSDVP50_00091	+	71 (16)	4.48E-05	AAAATAGTAT	AATAATGAAATCAAA	ATACCTAATA
PAPL	+	20 (66)	6.03E-05	GTGGATTTGT	AATAATAAAAAAAAA	TTATTTAAAA
LSDVP50_00031	+	42 (44)	7.66E-05	TTACTATAAC	ATAAATGAAAAACTA	TTATAAAAAA
F4L	+	65 (21)	1.07E-04	GACTAAATAA	AATAATGAAATATTA	ATTTTTATGG
LSDVP50_00016	+	69 (17)	3.36E-04	GTTTTTTATA	ATAAAGGAAAATATA	AGTTACTTTT
VITF3S	-	39 (47)	3.84E-04	TTTATACAGT	ATAAATAAAATGAAA	TATATTGTAA

NB: Bold nucleotides are the motif/ conserved region of the LSD promoter. The hierarchy of promoter motifs were placed on top according to their significance level (P-Value)

* Gap or spacer nucleotides between motif to initiation codon = Total number of nucleotide - (start point of motif-1) - number of nucleotide in motif - initiation codon

Here, Gap between motif to initiation codon = 103-(start point-1)-15-3

Discussion

The present study explores into the *in-silico* assessment of species-specific early gene putative promoter motifs within the Indian LSDV vaccine strain. Through a comprehensive computational analysis, we have unveiled several key findings that shed light on the potential regulatory mechanisms governing early gene expression in this important viral pathogen.

Our analysis identified a highly conserved 15 nt motif, 'AAAAATGAAAAAAAA,' which was consistently present in all 34 of the examined LSDV early gene promoters. This remarkable degree of conservation strongly implies the pivotal role of this motif in governing gene expression within the virus. Previously, the 7.5 kDa early gene promoter of VACV, a 16 nt motif, 'AAAAgTaGAAAataTA', has been identified as a critical determinant with substantial impact on gene expression (Davison and Moss, 1989) [2]. Furthermore, Yang *et al.*, conducted a temporal transcriptome analysis of VACV using deep RNA sequencing, leading to the discovery of an early promoter motif characterized by a 15 nt consensus sequence 'AAAANTGAAAANNNA' that is specific to VACV (Yang *et al.*, 2011b) [16]. The prevalence of Adenine-

rich motifs in the LSDV early gene promoters strongly implies their potential involvement in facilitating gene expression. These motifs likely act as binding sites for transcription factors, suggesting a role in recruiting essential components of the transcriptional machinery or regulatory proteins. The specific structural characteristics of Adenine-rich motifs, such as favourable hydrogen bonding and base pair flexibility, may contribute to stable interactions with transcription factors, thereby influencing the initiation of gene expression in LSDV.

Understanding the distribution and proximity of these motifs relative to the TSS is pivotal for enhancing the efficacy of recombinant vaccines and for optimizing gene expression within the context of the LSDV vector system. Our analysis revealed that the putative promoter motifs are predominantly situated within the middle region of the promoter sequence. This central localization suggests that the regulatory elements responsible for initiating transcription are concentrated within this region. The analysis also highlighted a notable variation in the proximity of the putative promoter motifs to the TSS. The minimum distance observed was 9 nt, as in the case of LSDVP50_00081, and the maximum distance was 66 nt, observed in early promoter PAPL. Variability in the distances between the promoter motifs and the TSS suggests a flexible regulatory system within the virus and possible variations in the transcription initiation strength among different genes within the early gene cluster. A closer motif-TSS distance may result in more rapid and efficient gene activation, while a more substantial gap may introduce additional regulatory complexity, or conversely. Di Pilato *et al.* (2015) [3] revealed the role of spacer length in VACV gene expression, using recombinant attenuated modified vaccinia Ankara (MVA) strains expressing GFP or Leishmania LACK antigen. Longer spacers correlated with increased early expression and enhanced T-cell (CD-4 and CD-8) responses, emphasizing the need for optimized spacer lengths in poxvirus-based vaccines. However, Alharbi *et al.* (2015) [1] found that inserting a 550-nucleotide IRES sequence as a spacer in rMVA did not enhance transgene expression or immunogenicity. These contrasting results highlight the complexity of optimizing spacer lengths in poxvirus-based vaccines, emphasizing the necessity for context-specific considerations in their design. By manipulating the position and strength of putative promoter motifs, researchers can potentially fine-tune the expression of protective antigens from other cattle pathogens within the LSDV genome.

Conclusion

In conclusion, this study lays the groundwork for future experimental validation of the identified promoter motifs within the Indian LSDV vaccine strain, shedding light on their functional significance. The discovered motifs hold potential for the creation of potent synthetic promoters specific to LSDV, while the observed variations in spacer lengths between the TSS and regulatory core motifs across early gene promoters may have critical implications for gene expression dynamics during early infection. This research sets the stage for further experimental validation and fine-tuning of promoter elements, promising to optimize the efficacy of LSDV-based recombinant vectored vaccines for comprehensive cattle disease control.

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