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Cloning and sequencing of lipoprotein signal peptidase (LSP) gene of *Anaplasma marginale*

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Abstract
An enzyme known as Lipoprotein signal peptidase (LSP), is essential for the production of lipoproteins. LSP is responsible for cleaving the signal peptide from preprolipoproteins, allowing their proper targeting and insertion into the cell membrane. The sequence of the peptidase was inferred from the nucleotide sequence of the *Anaplasma marginale* LSP gene, whice codes for lipoprotein signal peptidase. The effectiveness of lipoprotein signal peptidase (LSP) inhibitor, against the *Anaplasma* genus of Gram-negative bacteria is demonstrated. The study chooses to target LSP, the enzyme in charge of digesting lipoproteins and determine the effect on *Anaplasma* survivability. Basically the truncated LSP gene of *Anaplasma marginale* is cloned and sequenced in this current study.

Keywords: Lipoprotein signal peptidase, *Anaplasma marginale*, cloning, sequencing

Introduction
The gram-negative bacteria *Anaplasma marginale*, which belongs to the order Rickettsiales and the family Anaplasmataceae, causes the arthropod-borne hemoparasitic illness anaplasmosis in cattle and other ruminants [1]. The disease is characterized by severe anemia associated with intra erythrocytic parasitism, resulting in fever, depression, and weakness [2]. The disease persists in clinically recovered cattle, serving as a reservoir from which it might transmit to unaffected cattle. Due to anaplasmosis, the production of meat, milk, and fibre is significantly decreased in tropical and subtropical parts of the world [3]. Bacterial lipoprotein biosynthesis is an attractive target for novel antibiotic drug discovery, as bacterial lipoproteins play critical roles in adhesion, nutrient uptake, antibiotic resistance, virulence, invasion, and immune evasion [4]. Lipoprotein biosynthesis in Gram-negative bacteria is mediated by three essential inner membrane localized enzymes, which work sequentially to generate the mature triacylated lipoproteins [5]. The major route of bacterial protein secretion from the cytoplasm is the Sec pathway [6]. Bacterial proteins that are transported by the Sec translocon are formed as preproteins with an amino-terminal extension known as the signal or leader peptide. The signal peptide is necessary for preprotein localization to the membrane for translocation by Sec machinery. During the translocation process, the signal peptides of non-lipoproteins are cleaved off by the type I signal peptidase (SPase I), while those of lipoproteins are cleaved off by the type II signal peptidase (SPase II). Thus, SPases play a key role in the transport of proteins across membranes in all organisms [7]. The LSP gene of *A. marginale* is cloned and sequenced in the present study.

Materials and Methods
Collection of Blood samples and isolation of Genomic DNA isolation
The bovine blood sample positive for *A. marginale* was collected from Unit of Veterinary Clinical Complex, FVAS, BHU. The sample was collected in an EDTA vials. 200 microlitre blood sample was used for isolation of whole genomic DNA. Following the instructions provided by the Gene JET Genomic DNA Purification Kit, the DNA was isolated. By using UV absorbance and electrophoresis on a 1% agarose gel, the purity and concentration of the genomic DNA were assessed.

PCR amplification of LSP gene
Manually designed primers for the LSP gene were custom synthesized. The primers' Tm was

calculated (Melting Temperature) while taking into account each base pair's property and checking for compatibility. The restriction enzyme sites for KpnI and NcoI, which were introduced at the 5' end of forward and reverse primers, respectively. (Table 1) contains the primer sequences. The extracted genomic DNA served as the template for PCR. Master Mix-60 µl, 6 µl of forward primer, 6 µl of reverse primer, and 5 µl of extracted genomic DNA were added to the 120 µl reaction mixture for 6 tubes following the protocol as described earlier [8, 9]. The final volume was adjusted using nuclease-free water (PROMEGA), followed by a short spin and allocation of 20 µl to each of the 6 PCR tubes. According to the following reaction conditions, the reaction was conducted in a thermal cycler (Applied Biosystem) up to 28 cycles: where the initial denaturation was at 95 °C for 5 minutes, denaturation at 95 °C for 30 sec, annealing at 53 °C for 30 sec, extension at 72 °C for 30 sec, and final extension at 72 °C for 10 minutes. The PCR product was identified by using Gel electrophoresis on 1% agarose gel with eithidium bromide (10 µl) under UV illumination.

Table. 1: Sequence of primers used for the amplification of LSP gene of *Anaplasma marginale.*

Primer Name	Sequence	Target
LSP Forward primer	ATCCATGGGGATCGATTGGTACTCACAATC	Amplification of LSP gene of *A. marginale.*
LSP Reverse primer	ATGGTACCTTAATATTTCTTAACGGAGAGC	

Cloning and sequencing of LSP gene

The PCR product was gel eluted accordingly using the recommended protocol of the (Thermo Scientific) GeneJET Gel Extraction kit (Cat. No. K0691). The PCR product as well as the vector PproExhta were both restriction digested with KpnI for 30 minutes followed by NcoI for 15 minutes at 37 °C. The Restriction Endonuclease enzymes were inactivated by incubation period for 10 min at 80 °C. By using the Gene JET Gel extraction kit (Cat. No.-k0691) of (Thermo Scientific), the digested PCR products and PproExhta were gel purified. The ligation reaction was carried out by using T_4 DNA ligase (PROMEGA). An mixture of insert and vector with 3:1 ratio was obtained, which was given a short spin and then it was followed by overnight incubation at 4 °C. 10 µL of this mixture having LSP insert was transformed into the chemically competent BL21 cells following 30 minutes transformation protocol as described by (Mishra *et al*., 2020) [10, 11]. The transformed cells were spread out by L spreader on Amp^+ (30 µg/ mL) LB plates and was incubated at 37 °C for overnight. Colony PCR was utilised for the screening of positive colonies. The recombinant plasmid containing the LSP insert was isolated by (MACHEREY-NAGEL GmbH & CO.KG) DNA, RNA and Protein Purification Kit (Nucleospin Plasmid) according to the manufacturer's instruction and was confirmed by PCR amplification, agarose gel electrophoresis, insert release after RE digestion and sequencing. The data was provided from custom sequencing of LSP gene was scrutinized by various software and aligned by EMBOOS needle. As a result of the query against the sequence of LSP gene of *Anaplasma marginale* is provided.

Results and Discussion

The whole blood genomic DNA was isolated with utmost purity and concentration. The PCR amplified product was found to be of desired length i. e. 438 bp (Fig. 1). After transformation, the positive clones were selected on an Amp+ plate (Fig. 2). After an overnight incubation, we can see positive transformants produces a few white translucent, recombinant colonies. The cloned were further harvested and confirmed for the insert by colony PCR with the vector specific primers (Fig. 3a), PCR with vector specific primers and isolated recombinant plasmids (Fig. 3b) and insert release (Fig. 3c). The aligned sequence of the LSP is presented in fig. 4.

Lipoprotein processing by SPase II has been shown to be necessary for the survival of gram-negative bacteria. These results suggest that the membrane-bound protease LSPA is a promising target for chemotherapy [12]. The annotating published rickettsial genome sequences revealed the existence of bacterial Sec translocon homologs [13]. However, the genes specifically involved in rickettsial protein secretion remain uncharacterized. In order to elucidate the mechanism of protein secretion and their role in rickettsial virulence, we are interested in characterizing the genes involved in the rickettsial Sec pathway. Lipoproteins have a significant role in the structural and functional makeup of bacteria [14] and the survival of these microorganisms depends on the manufacture of these proteins. The identification and characterization of specific genes and proteins of *Anaplasma marginale*, such as the lipoprotein signal peptidase (LSP) gene have been major focus of the study. These study provide insights into the virulence factors, prospective therapeutic targets, and diagnostic markers for the bacteria. Additionally, understanding the immune response of the host and developing vaccines against *Anaplasma marginale* has been a significant area of research to mitigate the impact of the disease on livestock.

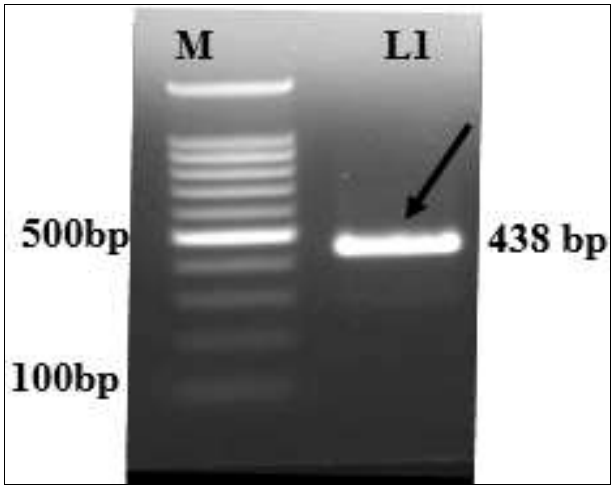


Fig 1: 1% Agarose gel electrophoresis of PCR product of LSP amplified from genomic DNA of *Anaplasma marginale* strain. M: 100 bp ladder; L1: PCR product.

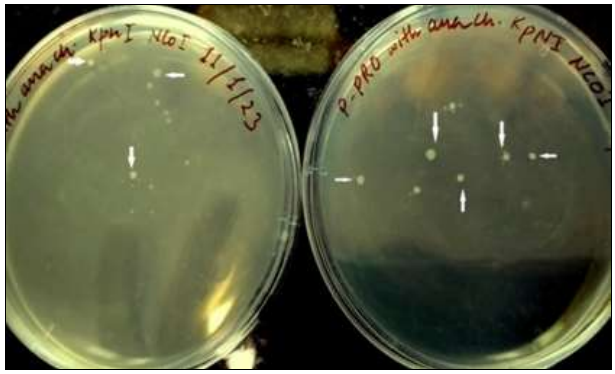
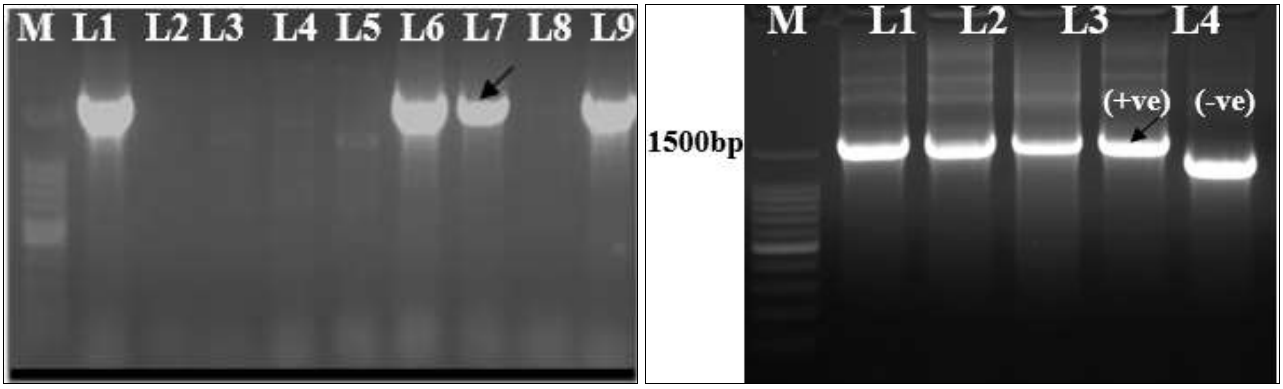
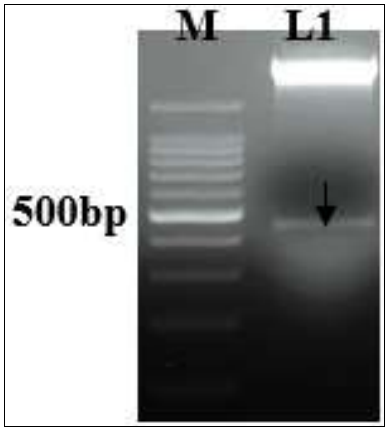


Fig 2: Transformed component cell BL21 colonies grown on Amp+ Luria-Bertani agar plates



a) b)



c)

Fig 3: Confirmation of LSP insert by (a) colony PCR, (b) vector specific PCR and (c) insert release. The products were analysed on 1 % agarose gel with ladder.

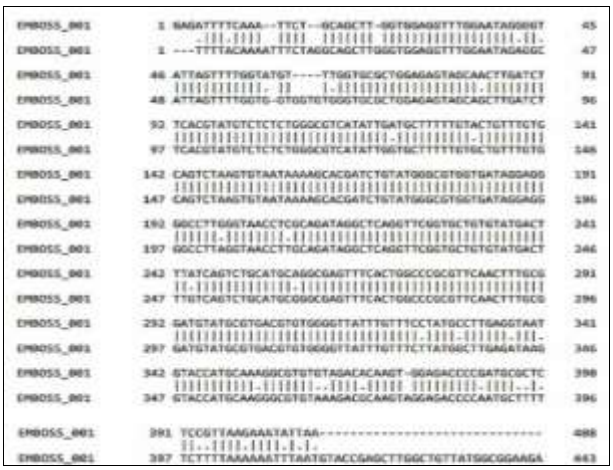


Fig 4: The sequence of the truncated LSP was aligned with the reference nucleotide sequence retrieved from NCBI GenBank [15].

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