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Molecular cloning of 1-396 BP of goat *Vitronectin* (VTN)

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Abstract
The extracellular matrix protein Vitronectin has immense importance in cell spreading, migration and adhesion. The roles are not confined to these rather it also regulates complement cascade and adhesion of bacterial pathogens. The molecule possesses various domains which renders it to be multifunctional. Goat vitronectin significantly differs from human vitronectin in possessing extra RGD sequences and absence of any signal sequence. In this study initial 396bp of goat VTN was cloned into a prokaryotic expression vector. The clones were further confirmed by colony PCR and gel retardation assays.

Keywords: extracellular matrix protein, vitronectin, cloning, RGD, prokaryotic, expression vector

1. Introduction
Goat vitronectin is a polymer of 444 amino acids encoded by 1332bp of coding sequences (Mahawar and Joshi, 2008) [2]. The presence of two RGDs at its N-terminus makes it distinct from other species. This extracellular matrix protein has several domains with unique functions. The initial 44 aa is denoted as Somatomedin B domain which is responsible in interaction with plasminogen activator receptor and urokinase PAR system (Preissner K.T. 1991) [4]. There are 4 hemopexin domains present in the molecule function of which is not yet established (Singh *et al.* 2011) [5]. Apart from these, three heparin binding domains (HBDS) indicate cationic nature of the protein. Role of goat vitronectin in binding to *Staphylococcus* (Mahawar and Joshi, 2008) [2] and complement C9 (Prasada *et al.* 2017) [3] has been characterized to C- and N- terminus of the molecule respectively. In the current study an attempt was made to clone the 1-396 bp of goat vitronectin (VTN).

2. Materials and Methods
2.1 Primer design
The coding sequences of goat VTN (with accession number DQ189087) was retrieved from NCBI gene bank. Gene specific primers were designed (table 1) manually and were checked by Oligo Analyzer 3.1 (https://eu.idtdna.com/calc/analyzer). The forward primer contained restriction endonuclease site for EcoRI whereas the reverse primer was added with HindIII restriction site.

Table 1: Primers used in the study

Primers	Sequence 5'-3'
Forward primer	ATGAATTCGACCAAGAGTCATGCAAGG
Reverse primer	ATAAGCTTCCCACTGCACGTTTCCTC

2.2 PCR amplification
Recombinant plasmid with 1-601bp of goat VTN was available in the lab and was used in the study for amplification of the desired fragment. Eppendorf Master Cycler Gradient PCR Thermal Cycler model 5331 was used for the amplification of the VTN fragment. The reaction mixture and conditions for the amplification are given in table 2 and 3 respectively.

Table 2: PCR conditions

Steps	Temperature	Time	
Initial Denaturation	95 °C	5 min	35cycles
Denaturation	95 °C	30 sec	
Annealing	56 °C	30 sec	
Extension	72 °C	45 sec	
Final extension	72 °C	10 min	

Table 3: PCR reaction mixture

Components	Volume used (µl)
PCR Master mix(2X)	12.5
Forward primer	1
Reverse primer	1
Template	1
NFW	9.5
Total	25

2.3 Plasmid Isolation
Prokaryotic expression vector pPROEXHTb (life technology) was used for the cloning of desired fragment. Plasmid was isolated from an overnight grown bacterial culture by using Qiagen plasmid isolation kit following the instructions from the manufacturer. The plasmid was quantified by nanodrop (ND-1000, Thermo Fischer Scientifics)

2.4 Restriction digestion and gel elution
1 µg of both plasmid and PCR amplicon were digested with restriction enzymes EcoRI and HindIII (New England Biolabs) for 1 hour at 37 °C (table 4). The digested PCR product and plasmid were electrophoresed in a 1% agarose gel. The digested products were gel eluted by Qiagen gel elution kit (Cat number: 28604)

Table 4: Restriction Digestion of plasmid and PCR amplicon

Plasmid: 15µl	PCR amplicon: 15µl
Eco RI: 1µl	Eco RI: 1µl
Hind III: 1µl	Hind III: 1µl
RE buffer (10X): 5 µl	RE buffer (10X): 5 µl
NFW: 28 µl	NFW: 28 µl
Total: 50 µl	Total: 50 µl

2.5 Ligation and transformation
The vector and insert were ligated at 1:3 molar ratio by using T4 DNA ligase (New England Biolabs). Competent *E coli*. (BL 21) cells were prepared by following the protocol described by Chung and colleagues (1989). About 50 ng of ligation mixture was added to 200 µl aliquot of competent cells followed by brief exposure to heat shock at 42 °C (45 sec). The suspension mixture was added with 1 ml of pre-warmed Luria Bertani broth (37 °C) and was incubated for 1 hour with constant shaking. The cells were pelleted and spread on Ampicillin (100µg/ml) containing LB agar plates. The plates were dried and were incubated at 37 °C for overnight.

2.6 Screening of colonies by Colony PCR
The colonies grown on Amp + plates were screened for 396 bp positive recombinant clones by 1-396 bp VTN specific primers. The PCR reaction was performed essentially as described before but 1 µl of suspension from colonies boiled in 20 µl of PBS were used as template. The amplicons were analysed on a 1% agarose gel.

2.7 Gel retardation assay
Plasmid was isolated from one of the colony PCR positive clones and subjected to agarose gel electrophoresis with a naïve pPROEXHTb plasmid. The retardation in migration pattern of the recombinant plasmid was compared with the naïve plasmid in a gel documentation system (Gel Doc^{TM} XR+ gel documentation system, Bio Rad Labs)

3. Results
The PCR amplified VTN fragment was analysed on a 1% agarose gel. The amplicon was found to be of near 400 bp (fig 1). The isolated plasmid upon gel electrophoresis shown 3 distinct bands (fig 2) which is in accordance with the usual plasmid characteristics on gel electrophoresis. The isolated plasmid was having a concentration of 65 ng/µl with $A_{260/280\ nm}$ of 1.92 which indicated purity of the plasmid. After transformation, countable numbers of fully grown colonies of BL 21 cells were seen on Amp+ positive LB agar plates (fig 3). All the seven colonies analysed were found to be positive for the insert (396 bp) in colony PCR (fig 4). Amplicons of near 400 bp size were amplified from all seven colonies. The isolated plasmid from the positive clone lagged behind the naïve pPPROEXHTb on agarose gel electrophoresis (fig 5).

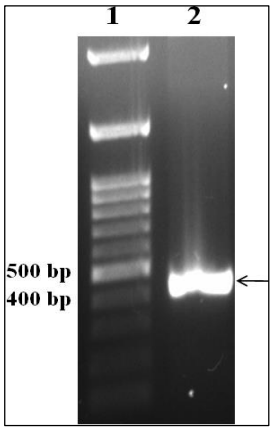

Fig 1: Agarose gel electrophoresis of PCR amplified 1-396 bp of caprine Vn. Lane 1: 100 bp DNA ladder and Lane 2: PCR amplified product

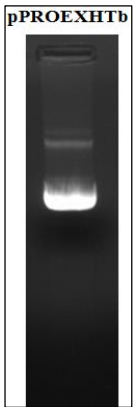

Fig 2: Agarose gel electrophoresis of pPROEXHTb prokaryotic expression vector

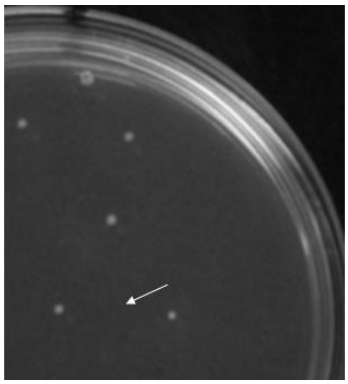

Fig 3: 1-396 bp VTN insert positive BL 21 colonies. Ampicillin was added to the selection media (LB agar) at 100 µg/ml

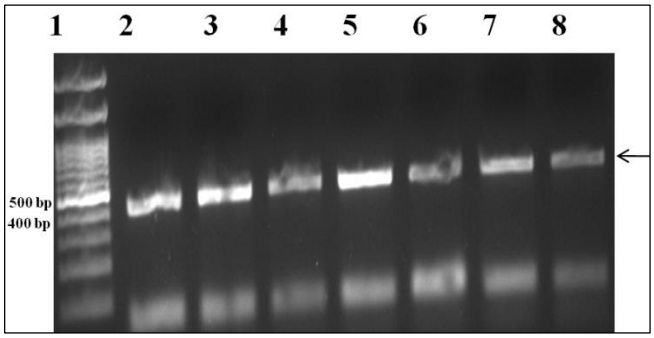

Fig 4: Screening of 396 bp positive colonies by Colony PCR. Agarose electrophoresis of Colony PCR amplified products. Lane 1: 100 bp ladder, Lane 2, 3, 4, 5, 6, 7 and 8 contains PCR amplified products

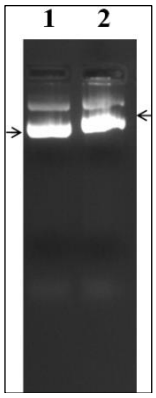

Fig 5: Gel retardation assay: Agarose gel electrophoresis analysis of (Lane 1) pPROEXHTb and (Lane 2) 396 positive pPROEXHTb

4. Discussion

The PCR amplicon was found to be little above the 400 bp which could be due to the additional sequences of RE sites included in the primers. With those additional sequences the amplicon was expected to be of 412bp. The colonies were screened with colony PCR as there is chance of incorporation of empty vector into the competent cells which will also show resistance against ampicillin. The additional 396 bp slowed the migration of recombinant plasmid as observed during electrophoresis. This result further confirmed the successful construction of the clone positive for 1-396 bp of goat VTN.

5. Acknowledgements

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6. References

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