Chapter 30

Laboratory Scale Production of Recombinant Haa86 Tick Protein in Pichia pastoris and in Escherichia coli System

Binod Kumar, Azhahianambi P., and Srikant Ghosh

1 Introduction

Hyalomma anatolicum, a tick vector for *Theileria annulata* responsible for bovine tropical theileriosis, is prevalent in many parts of the world and almost all over India and causes heavy economic loss to livestock sector [1]. As an alternative to acaricide, the immunological control of ticks was found to be an effective component of the integrated control of the tick species [2]. In the line of success of Bm86-based vaccines against *Rhipicephalus* (*Boophilus*) *microplus* (TickGARD™, TickGARD plus™, and Gavac™) [3–5], the Bm86 homologue of *H. anatolicum* was expressed in both prokaryotic and eukaryotic expression systems, and its efficacy against both homologous and heterologous challenge were recorded [6, 7].

Purification of native midgut antigen from ticks is tedious, laborious, time-consuming, and low-yielding procedure. Immunization trials in large animals followed by commercialization of vaccines necessitate the production of antigens in bulk quantities. Recombinant DNA technology using prokaryotic or eukaryotic expression systems have been utilized for the generation of targeted proteins in bulk. Both the above systems have certain advantage and disadvantage over each other (Table 1). Yeast offers site-specific integration, increase in copy number, leader sequence for the secretion of heterologous protein, posttranslational modifications, fast growth, and low-cost media [8, 9]. Similarly, *E. coli*-based expression system is well known for its simplicity, flexibility, and inexpensive expression of target protein. Moreover, extensive information of genetics and vast availability of compatible tools for genetic manipulation makes the system very popular [10].

Sunil Thomas (ed.), *Vaccine Design: Methods and Protocols, Volume 2: Vaccines for Veterinary Diseases*, Methods in Molecular Biology, vol. 1404, DOI 10.1007/978-1-4939-3389-1_30, © Springer Science+Business Media New York 2016

Table 1
Advantages and disadvantages of prokaryotic and eukaryotic expression system

Prokaryotic expression system	Eukaryotic expression system
Advantages	Advantages
• Easy cloning and genetic manipulation	• Post-translational modifications are possible
• Inexpensive to culture	• Protein secreted in medium can easily be purified
• Rapid growth and fast expression	• Rapid growth and high expression
• Flexible in expression (multiple promoters, tags, fusion proteins, cleavage site, etc.)	
• Usually work well for intracellular proteins	
• Can be optimized for soluble expression vs inclusion bodies	
Disadvantages	Disadvantages
• Unavailability of eukaryotic post-translational modifications	• Comparatively longer time required for selection of high-expression clone
• Lack of some tRNA common to eukaryotic genes which severely limits the expression and necessitates codon optimization of the eukaryotic gene to be expressed	• Inability to perform certain complex posttranslational modifications, such as prolyl hydroxylation and amidation as well as some types of phosphorylation and glycosylation
• Difficult to express the gene of interest as secretory protein	• Over glycosylation
• Formation of inclusion bodies and failure of proper folding of some proteins	

1.1 Importance of Hyalomma anatolicum

The H. anatolicum tick parasitize domestic (cattle, buffalo, sheep, and goat) and wild mammals and are abundant in semiarid zones of Asia, Near and Middle East, Southeastern Europe, and North Africa. In India, *H. anatolicum* has been incriminated as principal vector of *Theileria annulata*, *T. buffeli*, and *T. lestocardi* (*T. hirci*) in cattle, buffalo, and small ruminants [1, 11]. Besides *Theileria* species, the vector is responsible for transmitting human diseases and the subject has recently been reviewed [12]. The tick species follows a three-host life cycle under natural conditions, but under laboratory condition, (on rabbit-calf model) it follows two-host life cycle [13]. The tick species was established as homogenous (GenBank accession no. HM176656) *T. annulata* free acaricide susceptible reference tick line, IVRI line-II (national registration no. NBAII/IVRI/HA/1/1998), was maintained in rabbit-calf model, and was used as starting material. The major disadvantage of managing tick vectors by the application of chemical acaricides is the development of acaricide-resistant isolates [14].

1.2 Haa86

The Haa86 is a homologue of Bm86 in *H. anatolicum*. The Haa86 is a 657 amino acid long protein (1971 bp long gene, EU665682) having seven complete EGF-like domains. The identity of the EGF-like domains (domain 1–7) of Haa86 protein with the corresponding EGF-like domains of Bm86 was 78.3 %, 56.8 %, 60.9 %, 51.3 %, 62.2 %, 69 %, and 65.8 %, respectively. The amino acid sequence homology between Haa86 and Bm86 (Austrian strain) is 62.6 %. The N-terminal region of the protein has a 48 amino acid long putative signal sequence and C-terminal has a 32 amino acid anchoring sequence. Glycosylation of the Haa86 protein was confirmed by silver nitrate staining (Glycoprotein staining) [7].

1.3 Eukaryotic Expression Host (Pichia pastoris) and Vector (pPICZαA)

Pichia pastoris is widely used as an expression host for the production of a variety of intra- and extracellular recombinant proteins. The expression vector used in *P. pastoris* is based on the strong, tightly regulated promoter of the *P. pastoris* major alcohol oxidase gene (*AOX*) [15, 16]; has bacterial plasmid elements, pUCori, for propagation and amplification of plasmid into bacteria; has different elements like α-factor secretion signal for efficient secretion of recombinant protein and 5′AOX1 promoter for plasmid integration to AOX1 locus of yeast chromosome; and allows methanol-inducible high-level expression of the gene of interest. The C-terminal polyhistidine (6 × His) tag present in the vector is helpful for the purification of protein with metal-chelating resin and detection of protein using anti-histidine antibodies. Other genes associated with the yeast vector are Zeocin™ resistance (marker) gene and transcription termination sequence. The vector is integrated into the host chromosome which provides mitotic stability in the absence of selection.

1.4 Prokaryotic Expression Host (Escherichia coli) and Vector (pET32a)

To express the eukaryotic protein, normally *E. coli* and its compatible plasmid vector are the first method of choice, available with many molecular tools and protocols. For instance, lists of expression plasmids, a large number of engineered *E. coli* strains, and many cultivation strategies make the *E. coli* a perfect host to express the heterologous proteins [17]. However, many times expressed protein does not fold properly and form aggregates called inclusion bodies inside the *E. coli*. To avoid this happening, various strategies were used like expression at low temperature for short period of time or expression with highly soluble partner, such as thioredoxin (Thx), glutathione-S-transferase (GST), maltose-binding protein (MBP), etc., to improve the solubility of the expressed protein.

pET is a commercial prokaryotic expression vector (Novagen, USA) which was originally constructed by Studier and colleagues [18–20]. The pET32a is one of the important vectors of the pET series widely used in prokaryotic expression system. The vector possess a T7 promoter and terminator, thioredoxin (Trx) tag sequence, His-tag sequence, lac1 gene, pBR322 and f1 origin,

multiple cloning sites (MCS), ampicillin resistance gene, etc. It has many advantages over other expression vectors; the target gene is tightly regulated under strong T7 promoter for the transcription of gene and the leaky expression of T7 RNA polymerase was tightly regulated in host strain, *E. coli* BL21(DE3)PLysS, through production of small amount of T7 lysozyme. The T7 lysozyme acts as inhibitor of T7 RNA polymerase at low level [21]. A 109 amino acid length thioredoxin protein is expressed along with the target protein which improves the protein folding and solubility through disulfide bond formation and self-solubility. The *E. coli* strain NovaBlue or DH5α cells are used for the initial cloning of target DNA into pET vectors and for maintaining plasmids because they are *recA*$^-$ *endA*$^-$ and have high transformation efficiencies and good plasmid yields. For the expression of recombinant, protein cloned in pET vector must be transformed into *E. coli* strain containing a chromosomal copy of the gene for T7 RNA polymerase like BL21(DE3), BL21(DE3)PLysS, etc. These hosts are lysogens of bacteriophage DE3, a lambda derivative that has the immunity region of phage *21* and carries a DNA fragment containing the *lacI* gene, the *lacUV5* promoter, and the gene for T7 RNA polymerase [17, 22]. The flow diagram of expression of targeted protein in heterologous system is presented below (Fig. 1).

2 Materials

2.1 Tick (H. anatolicum)

1. *H. anatolicum* reference strain (*see* **Note 1**).
2. Clean 25 ml collection vial.
3. Tick washing solution (3 % H_2O_2, 70 % ethanol, distilled water).
4. Electronic weighing machine.
5. Deep freezer (−20 and −80 °C).

2.2 RNA Isolation and cDNA Preparation

1. Diethylpyrocarbonate (DEPC)-treated mortar and pestle and 0.5, 1.5 and 2.0 ml Eppendorf tubes.
2. Nuclease-free filter tips (10, 200, 1000 μl) and micropipettes.
3. Liquid N_2.
4. Reagent for RNA isolation: TRIzol™ Reagent (Invitrogen, USA), Molecular grade chloroform and isopropyl alcohol (Amresco, USA), nuclease-free water (NFW) (Ambion, USA), 70 % ethanol in nuclease-free water (mix 35 ml molecular grade absolute alcohol (Merck, Germany) in 15 ml NFW present in 50 ml nuclease-free tube), and RNaseZap® (Ambion, USA).
5. Tabletop refrigerated centrifuge (Hermle, Germany).
6. Spectrophotometer (NanoDrop) (Thermo Scientific, USA).

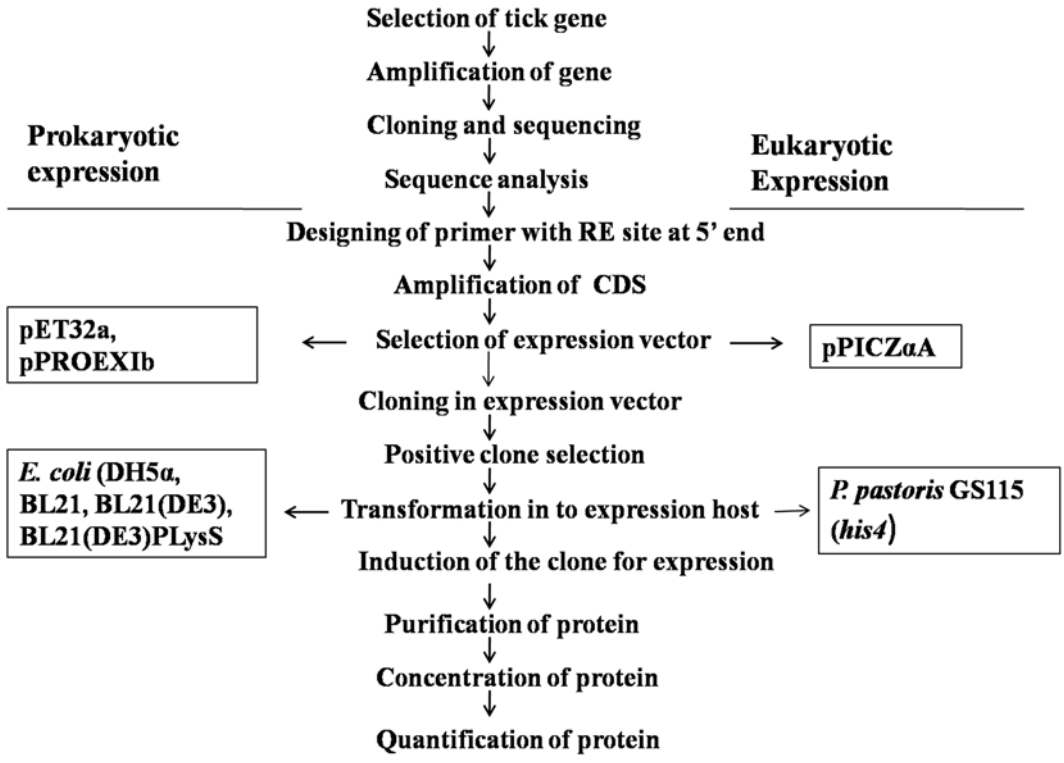

Fig. 1 Flow diagram of rHaa86 protein production

7. cDNA preparation reagent: RevertAid H Minus First Strand cDNA Synthesis Kit, (MBI Fermentas, USA) containing reverse transcriptase (RT), RNase inhibitor, oligo-dT, RT buffer, dNTPs, and NFW.
8. Temperature-controlled dry bath/water bath (Genei, India).
9. Deep freezer (−20 and −80 °C).

2.3 Cloning and Sequencing

1. PCR workstation, 0.2 ml PCR tubes, and PCR machine.
2. PCR reagents: 10× PCR Buffer, 10 mM dNTPs, Hot Start Taq DNA Polymerase (MBI Fermentas, USA), cDNA, NFW, and custom synthesized forward (HA1) and reverse primer (HA2) at working dilution of 10 μM conc. in NFW [HA1—5′CGGC GGATCC TTG TTC GTT GGC GCT ATT TTG CTC AT 3′ and HA2—5′CCC GGTACC TCTAGA TGC AAC GGA GGC GGC CAG TAA 3′].
3. Agarose (Amresco, USA), 6× loading dye and GeneRuler™100 DNA Ladder Plus (MBI Fermentas, USA), and ethidium bromide (SRL, India) suitable for gel electrophoresis.

4. Agarose gel electrophoresis: for 25 ml of 1.0 % agarose gel, use 250 mg of ultrapure agarose (electrophoresis grade) with 25 ml of 1× TAE. Prepare 500 ml of 50× TAE stock solution in ultrapure water with 121 g of Tris base, 50 ml 0.5 M disodium EDTA (pH 8), and 28.55 ml glacial acetic acid.
5. Electrophoresis system with power pack (Applied Biosystem, USA).
6. Gel Documentation System (Syngene, UK).
7. Laminar air flow cabinet, tabletop centrifuge, and NanoDrop spectrophotometer.
8. QIAquick Gel Extraction Kit (Qiagen, Germany).
9. InsTAclone PCR Cloning Kit (MBI Fermentas, USA): includes vector (pTZ57R/T), 5× ligation buffer, T4 DNA ligase, NFW, *E. coli* growth media (C-media), and reagent for competent cell preparation (T-Sol A and T-Sol B).
10. *E. coli* strain DH5α (F^- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17(r_{K^-} m_{K^+}), λ–), culture plate, spreader, X-gal (20 mg/ml, MBI Fermentas, USA), 1 M IPTG (MBI Fermentas, USA) (dissolve the 23.83 mg IPTG in 1 ml of distilled water, filtered through the 0.22 mm syringe filter and stored at –20 °C), and ampicillin (MBI Fermentas, USA) (100 mg/ml), dissolve 100 mg of ampicillin Na in 1 ml of distilled water, filtered through the 0.22 mm syringe filter and stored at –20 °C.
11. Agar plate: Dissolve the Luria-Bertani (LB) agar (Amresco, USA) in 50 ml of distilled water (for two plate) and mix well by heating and autoclave for 15 min. Dissolve 100 μl of X-gal, 25 μl of 1 M IPTG, and 50 μl ampicillin after cooling the autoclaved agar up to 50–60 °C. Pour the contents in presterilized culture plate. After solidification of agar, it can be immediately used or stored at 37 °C for 24–48 h covered in aluminum foil.
12. Stab culture tube: Add 1 ml of autoclaved LB agar mixed with ampicillin in sterile 1.5 ml Eppendorf tube, cool at room temperature and at 4 °C for 1 week.
13. DNA sequencing facility.

2.4 Sequence Analysis and Design of Expression Primer for Targeted Gene

1. GeneTool, DNASTAR software.
2. National Centre for Biotechnology (NCBI) BLASTn online server.

2.5 Expression of Haa86 in Pichia pastoris

1. PCR workstation, 0.2 ml PCR tubes, and PCR machine.
2. PCR reagent: 10× PCR Buffer, 10 mM dNTPs, Hot Start Taq Polymerase (MBI Fermentas, USA), template DNA (pTZHA86), NFW, and custom synthesized forward (HA3) and reverse primer (HA4) with suitable RE site (BamHI and

XbaI, respectively) at working dilution of 10 μM conc. in NFW [HA3—5′CGGC GGATCC GGT AGA GAG GAT GAT TTC GTG TG 3′ and HA4—5′CCC TCTAGA GTCGAC TGT TGC TTC TGT AGT TGT TGC TTC T 3′].

3. Agarose (Amresco, USA), 6× loading dye and GeneRuler™100 DNA Ladder Plus (MBI Fermentas, USA), and ethidium bromide (SRL, India) suitable for gel electrophoresis.
4. Agarose gel electrophoresis (*see* Subheading 2.3, **item 4**).
5. Agarose gel electrophoresis system with power pack (Applied Biosystem, USA).
6. Gel documentation system (Syngene, UK).
7. Laminar air flow cabinet, tabletop centrifuge, and NanoDrop spectrophotometer.
8. Restriction enzymes (RE): BamHI, XbaI, EcoRI, NotI, and PmeI and 10× buffer [Yellow Tango, Buffer (B+), Buffer (NEB), Orange (O+) buffer] (MBI Fermentas, USA).
9. Vector: Prokaryotic expression vector pPROEXHTb (Invitrogen, USA), prokaryotic cloning vector pBluescript II KS (+) (MBI Fermentas, USA), and yeast expression vector pPICZαA (Invitrogen, USA).
10. T4 DNA ligase and 10× ligation buffer (MBI Fermentas, USA).
11. *E. coli* DH5α competent cells (Invitrogen, USA).
12. Culture plate, spreader, X-gal (20 mg/ml, MBI Fermentas, USA), 1 M IPTG, and ampicillin (100 mg/ml) (*see* Subheading 2.3, **item 10**).
13. Agar plate (*see* Subheading 2.3, **item 11**).
14. Bacteriological and BOD incubators.
15. LB broth (Amresco, USA): dissolve 2.5 g of LB powder in 100 ml of distilled water and autoclave.
16. Shaker incubator.
17. SDS-PAGE system and reagents (30 % Acrylamide/Bis-acrylamide solution (29:1)), 10 % sodium dodecyl sulfate (SDS), 10 % ammonium persulfate (APS), 1.5 M Tris-HCl (pH 8.8), 1 M Tris-HCl (pH 6.8), and TEMED (Sigma, USA).
18. Running buffer: Dissolve 0.2 g SDS, 1 g Tris, and 14.5 g glycine in 1000 ml of distilled water.
19. Staining solution: Dissolve 0.02 mg Coomassie Brilliant Blue R250, 300 ml of methanol, and 100 ml of glacial acetic acid in 600 ml of distilled water and filter through grade 4 filter paper and store in amber colored bottle.
20. Destaining solution: Add 300 ml of methanol and 100 ml of glacial acetic acid in 600 ml of distilled water and store at room temperature.

21. Zeocin® (Invitrogen, USA) 100 μg/ml added LB agar (low salt, 0.5 %) plate.
22. QIAprep spin Miniprep Kit and QIAquick Gel Extraction Kit (Qiagen, Germany).
23. Mutant methylotrophic yeast strain: *Pichia pastoris* GS115 (*his4*) (Invitrogen, USA).
24. YPD (yeast extract peptone dextrose) agar medium (SRL, India).
25. Ice cold autoclaved distilled water and ice cold sterile 1 M sorbitol.
26. 0.2 cm sterile electroporation cuvette (Bio-Rad) and electroporation apparatus (Bio-Rad).
27. YPDS (yeast extract peptone dextrose sorbitol) agar plates containing 100 μg/ml of Zeocin® (Phleomycin D).

2.6 Culture and Purification of Yeast Expressed rHaa86

1. Glycerol stock of positive *P. pastoris* Haa86 clone.
2. Sterile BMGH (buffered minimal glycerol with histidine): To prepare 100 ml of medium, add 1.0 g yeast extract and 2.0 g peptone in 68.75 ml of distilled water, mix well, and autoclave for 15 min. After cooling add 10 ml each of 1 M potassium phosphate buffer pH 6.0, 10× yeast nitrogen base (34 g YNB and 100 g ammonium sulfate in 1000 ml of distilled water), and 10× glycerol (dissolve 100 ml glycerol in 900 ml of distilled water, autoclave for 15 min), 1 ml 100× L-histidine (dissolve 400 mg histidine in 100 ml distilled water, filter through 0.2 μ filter), and 250 μl 500× biotin (20 mg biotin in 100 ml distilled water, filter through 0.2 μ filter). BMGY (buffered glycerol-complex medium) is similar to BMGH medium without histidine.
3. Sterile BMMH (buffered minimal methanol with histidine) medium: To prepare 100 ml of medium, add 1.0 g yeast extract and 2.0 g peptone in 77.75 ml of distilled water, mix well, and autoclave for 15 min. After cooling add 10 ml each of 1 M potassium phosphate buffer pH 6.0 and 10× YNB, 1 ml absolute ethanol, 1 ml 100× L-histidine, and 250 μl 500× biotin.
4. Disruption buffer 1 (DB1) containing 50 mM sodium phosphate, 5 mM EDTA, 10 % sucrose, 0.3 M NaCl, and 2 mM 2-mercaptoethanol, pH 7.0.
5. Sonicator.
6. Disruption buffer 2 (DB2) containing 50 mM sodium phosphate, 5 mM EDTA, 0.5 % Triton X-100, 0.3 M NaCl, and 1 mM 2-mercaptoethanol, pH 7.0.
7. Washing buffer (WB) containing 50 mM sodium phosphate and 5 mM EDTA, pH 7.0.
8. Pre-extraction buffer (PEB) containing 50 mM sodium phosphate, 5 mM EDTA, and 0.8 M urea, pH 7.0.

9. Extraction buffer (EB) containing 50 mM sodium phosphate, 5 mM EDTA, 8 M urea, and 2 mM 2-mercaptoethanol, pH 7.0.
10. Refolding buffer (RB) containing 25 mM Na_2HPO_4 and 1.5 mM EDTA, pH 10.5.
11. 50 kDa cut off ultrafilter (PALL life sciences).
12. 1 N HCl.
13. SDS-PAGE system and reagents (*see* Subheading 2.5, **item 17** and **18**).
14. 25 % isopropyl alcohol, 7.5 % and 10 % acetic acid, and 0.2 % aqueous periodic acid in distilled water.
15. Freshly prepared filtered ammoniacal silver solution (100 ml containing 1.4 ml NH_4OH, 21 ml 0.36 % NaOH, and 4 ml 19.4 % $AgNO_3$).
16. Freshly prepared destaining solution (0.005 % citric acid, 0.019 % formaldehyde solution, and 10 % methanol).

2.7 Expression of Haa86 in E. coli

1. PCR work station, 0.2 ml PCR tubes, and PCR machine.
2. Haa86 positive clone (TA-cloning vector containing Haa86 insert).
3. PCR reagent: 10× PCR buffer, 10 mM dNTPs, Hot Start Taq Polymerase (MBI Fermentas, USA), template DNA (pTZHA86), NFW, and custom synthesized forward (HA3) and reverse primer (HA4) with suitable RE site (EcoRI and XhoI, respectively) at working dilution of 10 μM conc. in NFW (*see* Subheading 2.5, **item 2**).
4. Agarose (Amresco, USA), 6× loading dye and GeneRuler™100 DNA Ladder Plus (MBI Fermentas, USA), and ethidium bromide (SRL, India) suitable for gel electrophoresis.
5. Agarose gel electrophoresis (*see* Subheading 2.3, **item 4**).
6. Electrophoresis system with power pack.
7. Gel documentation system, tabletop centrifuge, and NanoDrop spectrophotometer.
8. Restriction enzymes (RE): EcoRI, XhoI, and 10× Tango yellow buffer (MBI Fermentas, USA).
9. Prokaryotic expression vector pET32(a) (Invitrogen, USA).
10. T4 DNA ligase and 10× buffer.
11. *E. coli* NovaBlue and Bl21(DE3)PLysS competent cells (Invitrogen, USA).
12. Culture plate, spreader, 1 M IPTG, ampicillin (100 mg/ml), and chloramphenicol (34 mg/ml)—dissolve 34 mg chloramphenicol in 1 ml of absolute ethanol and store at −20 °C (*see* Subheading 2.3, **item 10**).
13. Agar plate with ampicillin (*see* Subheading 2.3, **item 11**), add only 50 μl ampicillin; do not use X-gal and IPTG.

14. Agar plate with ampicillin and chloramphenicol (*see* Subheading 2.3, **item 11**), add 50 μl each of ampicillin and chloramphenicol; do not use X-gal and IPTG.
15. Bacteriological incubator.
16. LB broth (Hi-Media, India): dissolve 2.5 g of LB powder in 100 ml of distilled water and autoclave.
17. Shaker incubator.
18. QIAprep Spin Miniprep Kit and QIAquick Gel Extraction Kit (Qiagen, Germany).
19. SDS-PAGE system and reagents (*see* Subheading 2.5, **item 17**).
20. 6× sample buffer and molecular weight protein marker (Genei, India).
21. Running buffer, staining, and destaining solutions (*see* Subheading 2.5, **items 18–20**).

2.8 Culture and Purification of *E. coli* Expressed rHaa86

1. Glycerol stock of positive Haa86 *E. coli* clone [pET32(a) Haa86-BL21(DE3)PLysS].
2. Two 250 ml conical flask with 50 ml LB broth in each (dissolve 2.5 g of LB powder in 100 ml of distilled water and autoclave for 15 min).
3. 1 M IPTG, ampicillin (100 mg/ml), and chloramphenicol (34 mg/ml) (*see* Subheading 2.3, **item 10** and Subheading 2.7, **item 12**).
4. Shaker incubator.
5. 50 ml centrifuge tubes and centrifuge machine.
6. Lysis buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris, pH 8.0).
7. Ni-NTA superflow resin (Qiagen, Germany).
8. Washing buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris, pH 6.3 and 5.9).
9. Elution buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris, pH 4.5).
10. Different concentration of urea (6, 4, 2 M) in PBS pH 7.2.
11. Autoclaved PBS pH 7.2.
12. 10 kDa cutoff dialysis bag and protein cutoff filter.
13. Cocktail protease inhibitor (Amresco, USA).

3 Methods

A 1965 bp gene fragment of Haa86 was amplified by PCR with primers HA1 and HA2. This fragment was cloned into pTZ57R/T vector to obtain the construct pTZHA86 (Subheading 3.3). Sequence length of 144 bp from 5′ end and 96 bp from 3′ end was deleted from the ORF of Haa86 by performing PCR with primers HA3 and HA4. The shortened Haa86 ORF with the size of 1755 bp was cloned, and the resulting plasmid construct was designated as pPROHA86. The 1755 bp Haa86 gene fragment was subcloned into pBluescript II KS (+), and the recombinant construct was designated as pBLHA86. The size of the insert released from the pBLHA86 was digested with EcoRI and NotI and calculated to be 1799 bp. Finally, the 1799 bp Haa86 gene fragment was subcloned into *P. pastoris* expression vector to obtain the construct pPICHA86 (Fig. 2) (Subheading 3.5). To express the protein in prokaryotic expression system, the 1755 bp ORF of Haa86 was amplified using HA3 and HA4 primer pair and cloned into pET32a vector. After positive selection of clone, the recombinant plasmid (pETHA86) was transformed into expression host *E. coli*-BL21(DE3)PLysS (Subheading 3.7).

3.1 Collection of Hyalomma anatolicum Unfed Adult Tick

1. Collect the engorged nymphs from animal and incubate in BOD incubator for 10–15 days (*see* **Note 1**).
2. Wash the hatched out adult tick first in 3 % H_2O_2, followed by 70 % ethanol, and finally in distilled water. Soak dry the water adhered to ticks using paper towel (*see* **Note 2**).

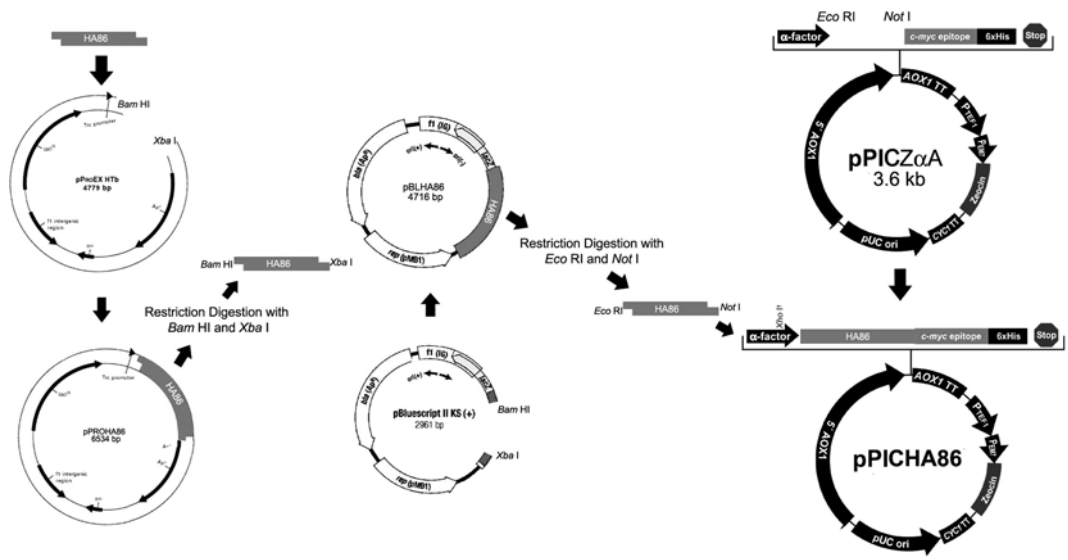

Fig. 2 Cloning strategies of Haa86 in eukaryotic expression vector (pPICZαA)

3. Weigh 50 mg of adult tick in 1.5 ml autoclaved tubes and store at −80 °C.

3.2 Whole RNA Isolation and cDNA Preparation

1. Day before RNA isolation, treat the mortar and pestle and 0.2, 1.5, and 2.0 ml tubes with 0.01 % DEPC in distilled water for overnight and autoclave (*see* **Note 3**). Wear gloves for entire process of RNA isolation.
2. Take out the preserved ticks from −80 °C. Immediately make a small hole on cap of tube and dip in liquid nitrogen hanging through a piece of thread for 5 min (*see* **Note 4**).
3. Apply the RNaseZap® around the workplace (*see* **Note 5**).
4. Quickly add liquid nitrogen-treated ticks into mortar and grind it with the help of pestle.
5. Add 1 ml TRIzol® reagent and mix well (*see* **Note 6**).
6. Collect the mixture in 1.5 ml tube and incubate at 4 °C for 30 min or −20 °C for overnight.
7. Centrifuge at 14,000 × *g* for 10 min at 4 °C and collect the upper aqueous phase into a new 1.5 ml tube kept on ice.
8. Add 200 μl of chloroform (0.2 volume of TRIzol) and vortex for 5–6 times (*see* **Note 7**).
9. Centrifuge at 14,000 × *g* for 10 min at 4 °C and collect the upper aqueous phase into a new 1.5 ml tube kept on ice.
10. Add 500 μl of isopropyl alcohol (0.5 volume of TRIzol) and mix gently. Incubate for 10 min at 4 °C or −20 °C for overnight (*see* **Note 8**).
11. Pellet the RNA, centrifuge at 12,000 × *g* for 10 min at 4 °C, and discard the liquid.
12. Wash the RNA pellet by adding 500 μl of 70 % ethanol in NFW, incubate for 5 min, and centrifuge at 10,000 × *g* for 2 min. Discard the liquid. Repeat this step for three times (*see* **Note 9**).
13. Air dry the RNA pellets and tube (*see* **Note 10**).
14. Add 50–100 μl of NFW, incubate on ice for 10 min, mix the RNA by mild tapping of the tube, and aliquot in 0.2 ml tubes.
15. Measure the concentration by taking OD at 260 nm using NanoDrop spectrophotometer and store at −80 °C.
16. Prepare the cDNA following the kit protocol (RevertAid H Minus cDNA synthesis kit, Thermo Scientific, USA) using oligo-dT primer.
17. Store the cDNA at −20 °C.

3.3 Cloning and Sequencing of Haa86 Gene

1. Custom synthesis of oligo primer based upon conserved region of tick gene (*see* **Note 11**).
2. Amplification of the targeted gene: For 25 μl reaction add 2.5 μl 10× Taq buffer, 0.5 μl 10 mM dNTPs, 1 μl each of 10 mM for-

ward (HA1) and reverse (HA2) primer, 1 μl (10–100 ng) cDNA, 0.3 μl Hot Start Taq Polymerase (5 unit/μl), and NFW to make 25 μl. Run the PCR as initial denaturation at 95 °C for 5 min and further 30 cycles at 94 °C for 1 min, 57 °C for 1 min, and 72 °C for 2 min and a final extension at 72 °C for 10 min.

3. Extraction of amplified product: Resolve the PCR product in 1 % agarose gel along with 100 bp plus DNA ladder, slice out the gel region containing targeted amplicons. Isolate the amplicons from gel slice following the protocol and reagent of gel extraction kit.
4. Quantify the amplicons concentration using NanoDrop spectrophotometer.
5. Setting of ligation reaction: Follow the guidelines of InsTAclone PCR Cloning Kit with slight modification. Typically for 30 μl reaction, add 3 μl vector (pTZ57R/T) and 5 μl gel-purified product in a 0.2 ml PCR tube and incubate at 65 °C for 5 min and then add 6 μl 5× buffer, 1 μl T4 DNA ligase, and 15 μl nuclease-free water. The ligation reaction is to be incubated at 4 °C overnight and then heat killed at 70 °C for 10 min (*see* **Note 12**).
6. Preparation of *E. coli* DH5α competent cells: Follow the guidelines of InsTAclone PCR Cloning Kit (*see* **Note 13**).
7. Transformation of ligated product into *E. coli* DH5α competent cells: Add 2 μl of ligation mix into 1.5 ml tube kept on ice and subsequently transfer 50 μl competent cell suspension in it. Incubate for 5 min on ice. Spread the transformed cell suspension on to the pre-warmed (37 °C) LB agar plate containing X-gal, IPTG, and ampicillin using sterile spreader under laminar air flow. After drying of surface moister, wrap the plate in aluminum foil and incubate at 37 °C upside down in a bacteriological incubator for overnight (*see* **Note 14**).
8. Select white bacterial colony from the plate having circular circumference (*see* **Note 15**).
9. Confirmation of the positive colonies by colony PCR: Take small part of the colony in 50 μl NFW present in 0.5 ml tube using bacteriological loop or 10 μl tip. Boil for 10 min in boiling water bath and centrifuge at 12,000 × *g* for 2 min. Use the supernatant as template DNA in a PCR reaction as stated above (*see* Subheading 3.3, **step 2**).
10. Growing of the colony in LB broth containing 100 μg/ml ampicillin: Left over colony (after colony PCR mix) is transferred to a 5 ml LB broth containing 100 μg/ml ampicillin (use 5 μl of stock ampicillin) present in 50 ml sterile tube. Incubate at 37 °C, 150 rpm for 5–6 h.
11. Preparation of stab culture: Dip 10 μl sterile tips in bacterial culture and pierce it into stab. Incubate at 37 °C for 12–16 h. Label it and send to DNA sequencing facility for nucleotide sequencing using 13 M forward and reverse primer. Positive recombinant plasmid can be designated as pTZHA86.

3.4 Sequence Analysis

1. Select the targeted sequence.
2. BLAST the sequence at BLASTn program of NCBI.
3. Select the coding DNA sequence (CDS) and identify the RE site within CDS.
4. Select the RE site(s) which are not present in CDS but available in multiple cloning sites (MCS) of the expression vectors.
5. Design the primer with different RE site(s) at 5′ ends of both forward and reverse primer.
6. Add 2–4 nucleotides upstream of the 5′ end before RE site.

3.5 Expression of Haa86 in Pichia pastoris

1. Amplification of Haa86 gene fragment (CDS) using primer HA3 and HA4: For 25 μl PCR reaction, use 2.5 μl 10× PCR buffer, 0.5 μl dNTP, 1 μl each of the primers HA3 and HA4, 1 μl template DNA (pTZHA86), 0.3 μl of Hot Start Taq Polymerase (MBI Fermentas, USA), and 18.7 μl NFW. PCR condition to be set as initial denaturation at 95 °C for 5 min and further 30 cycles at 94 °C for 1 min, 47 °C for 1 min, and 68 °C for 2 min and a final extension at 68 °C for 10 min.
2. Run the PCR product in 1 % agarose gel and purify the targeted amplicons using gel extraction kit.
3. Quantify the concentration of amplicons using NanoDrop spectrophotometer.
4. Double digest the vector pPROEXHTb and amplicons separately with BamHI and XbaI restriction enzymes. Typically set 50 μl reaction where 1 μg of vector and 2 μg of amplicons to be digested at 37 °C for 6 h in 1× Tango buffer by 2 unit each of RE.
5. Resolve the digested products separately in 1 % agarose gel and purify the digested products using gel extraction kit.
6. Setting up of ligation reaction for digested vector and amplicons: Add 10× ligation buffer (10 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 0.1 mg/ml BSA, 0.5 mM ATP), 70 ng of digested vector (pPROEXHTb), 200 ng of digested targeted gene, and 1 unit of T4 DNA ligase in 35 μl reaction volume. Incubate at 4 °C overnight and heat inactivate the enzyme at 70 °C for 10 min.
7. Prepare the *E. coli* DH5α competent cells (*see* **Note 13**).
8. Transform the ligated product in *E. coli* DH5α competent cells (*see* Subheading 3.3, **step 7**).
9. Screen the positively transformed cells from culture plate (*see* Subheading 3.3, **step 9**).
10. Extraction of the plasmid (pPROHA86): Grow the PCR positive colony (leftover) in a 5 ml LB broth containing 100 μg/ml ampicillin present in 50 ml sterile tube. Incubate in a shaking incubator

at 37 °C and 150 rpm for 4–5 h. Extract the plasmid using the protocol of QIAprep Spin Miniprep Kit and store at –20 °C.

11. Confirmation of positively transformed colony for expression of rHaa86: Grow the clone in 50 ml LB broth containing 100 μg/ml ampicillin (add 50 μl stock ampicillin) at 37 °C for 3 h. Subsequently, add 50 μl 1 M IPTG to it and continue the growth for next 4 h. Collect 1 ml culture and harvest the cell. Solubilize the harvested cells into 100 μl 1× sample buffer and boil for 10 min. After brief centrifugation, load 10 μl in SDS-PAGE. Observe the expressing colonies on gel after staining and destaining.
12. Releasing of gene insert from expressing pPROHA86 by BamHI and XbaI restriction enzymes: Set a 20 μl reaction contain 1 μg vector, 1× Tango buffer, and 1 unit each of BamHI and XbaI at 37 °C for 6 h. Purify the released product using gel extraction kit.
13. Digest the vector pBluescript II KS(+) with BamHI and XbaI restriction enzymes. Follow the above protocol.
14. Ligate the gene insert released from pPROHA86 and digested pBluescript II KS(+) (*see* Subheading 3.5, **step 6**).
15. Transform the ligated product into *E. coli* DH5α competent cells (*see* Subheading 3.3, **step 7**).
16. Screen the positively transform cells from culture plate (*see* Subheading 3.3, **step 9**).
17. Extract the plasmid (pBLHA86) from positive clone (*see* Subheading 3.5, **step 10**).
18. Releasing of gene insert from pBLHA86 by EcoRI and NotI restriction enzymes: Set a 50 μl reaction contain 2 μg vector, Orange (O+) buffer, and 1 unit each of EcoRI and NotI at 37 °C for 4 h. Purify the released product using gel extraction kit and quantify the concentration using NanoDrop spectrophotometer.
19. Digest the vector pPICZαA with EcoRI and NotI restriction enzymes. Follow the above protocol.
20. Ligate the gene insert released from pBLHA86 and digested pPICZαA (*see* Subheading 3.5, **step 6**).
21. Transform the ligated product into *E. coli* DH5α competent cells. Spread the transformed cells on to LB agar plate containing 100 μg/ml Zeocin® (*see* Subheading 3.3, **step 7**).
22. Screen the positively transform cells from culture plate (*see* Subheading 3.3, **step 9**).
23. Extract the plasmid (pPICZHA86) from positive clone (*see* Subheading 3.5, **step 10**).
24. Linearization of plasmid (pPICZHA86) by Pme 1 restriction enzymes: Set up 100 μl (25 × 4) reaction to linearize the pPICZHA86 with PmeI (MssI). Mix 20 μl vector (20 μg), 2 μl Pme1 enzyme (20 unit) and 10 μl 10× Buffer (B+) in 68 μl of nucle-

ase-free water. Incubate the reaction at 37 °C for 12 h. Purify the digested product using gel extraction kit (*see* **Note 16**).

25. Preparation of electrocompetent *P. pastoris* GS115 (*his4*): Inoculate 1 ml glycerol stock of *P. pastoris* GS115 (*his4*) into 10 ml YPD medium and incubate in orbital shaker (280 rpm/28 °C/16 h). Subculture into 200 ml of YPD medium by adding 2 ml starter culture in orbital shaker (280 rpm/28 °C) to attain 1.5 OD_{600}. Pellet the cells at 1500 × *g*/5 min/4 °C and resuspend in 200 ml of ice cold autoclaved distilled water. Again pellet the cell suspension and suspend in 20 ml ice cold sterile 1 M sorbitol. The cell suspension was kept on ice and immediately use for electro transformation (*see* **Note 17**).
26. Transformation of the linearized pPICZHA86 into electrocompetent *P. pastoris* GS115 (*his4*) cells: Collect the 80 μl cell suspension into an autoclaved 1.5 ml tube. Add 20 μl (7.5 μg) of the linearized pPICZHA86 and mix gently. Take this mixture into a sterile electroporation cuvette (0.2 cm) and incubate on ice for 5 min. Place the cuvette holder of the electroporation apparatus inside the laminar flow and fit the cuvette containing cells and DNA in it. Pulse the cells with a potential difference of 1.5 kV, resistance of 200Ω, and capacitance of 25 μF for 5 ms. Add 1 ml of ice cold sterile 1 M sorbitol into the cuvette immediately. Finally, incubate the cuvette at 30 °C for 1 h in BOD incubator without shaking.
27. Growing of transform cells on YPDS agar plates containing 100 μg/ml of Zeocin® (Phleomycin D): Spread 100 μl of the transformed cell suspension on the YPD agar plates containing 100 μg/ml of Zeocin® (Phleomycin D) and incubate the plate in BOD incubator at 30 °C for 72 h (Fig. 3).

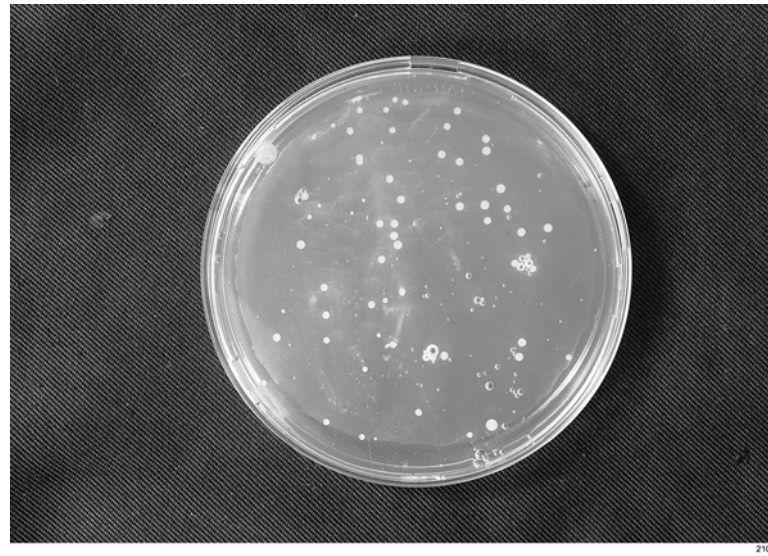

Fig. 3 Recombinant *Pichia pastoris* colony on YPD agar

28. Streaked some (10–15) of the good texture colony on new YPD agar plates containing 100 μg/ml Zeocin®. Streaked the colonies separately on the YPD agar plates containing 100 μg/ml Zeocin® and incubate in BOD incubator at 30 °C/48 h. Numbered the streaked colonies and store at 4 °C.
29. Screen 4–5 colonies for presence of insert through colony PCR (*see* Subheading 3.3, **step 9**).
30. Preparation of glycerol stock of the positive clone and storing at −80 °C: Culture the positively selected colonies into 10 ml BMGH media at 30 °C for 7 h and then add 1.7 ml autoclaved glycerol to it. Aliquot into 1 ml and store at −80 °C.

3.6 Purification of Yeast Expressed rHaa86 Protein

1. Growing of glycerol stock of positive clone(s) in basal minimum complex glycerol medium with histidine (BMGH): Inoculate 1 ml of *P. pastoris* HAA86 glycerol stock into 50 ml BMGH medium and grow in orbital shaker at 28 °C/230 rpm to reach 1.0 OD at 600 nm. Harvest the cell at 2000 × *g*/10 min/4 °C.
2. Growing in basal minimum complex methanol medium with histidine (BMMH): Resuspend the above harvested cells in 200 ml of BMMH medium and incubate in orbital shaker at 28 °C/230 rpm/120 h. Pellet the induced *P. pastoris* HAA86 culture at 14,000 × *g*/25 min.
3. Suspend the BMMH grown yeast pellet in disruption buffer 1 (DB1). Use 1.6 ml DB1 per gram of yeast pellet.
4. Sonicate the suspension at 10,000 Hz for 5 min interspersed with a period of cooling on ice. Pellet the cells by centrifugation at 20,000 × *g*/25 min.
5. Dissolve the pellet in disruption buffer 2 (DB 2) (4 volume of DB1) and incubate at 37 °C for 1 h on a shaker. Pellet the cells by centrifugation at 20,000 × *g*/25 min.
6. Pellet from DB2, dissolve in wash buffer (WB) (4 volume of DB2), and re-pellet it.
7. Dissolve the pellet in pre-extraction buffer (PB) (2 volume of wash buffer) and again re-pellet.
8. Suspend the pellet in WB (2 volume) again.
9. Finally, centrifuge to form pellet, dissolve into 2 ml of extraction buffer (EB).
10. Centrifuge at 30,000 × *g* for 25 min to collect the supernatant and store at −20 °C.
11. Add refolding buffer (RB) into the above supernatant after thawing. Dilute the supernatant with ten times of RB slowly for 8 h.
12. Concentrate the protein by 50 kDa cutoff ultrafilter. Collect the supernatant solution in cutoff filter and spin at 5000 × *g* for 50 min.

13. Precipitation of the contaminated yeast protein: Lower the pH of the supernatant solution to pH 4.8 by slowly adding 1 N HCl. Incubate at 4 °C for 15 min and precipitate the pellet at 16,000 × *g*/20 min/25 °C.
14. Concentrate the purified rHaa86 protein by 50 kDa cutoff ultrafilter.
15. Resolve the protein on 8 % SDS-PAGE.
16. Staining of the gel with silver nitrate stain to determine glycosylation: After electrophoresis, soak the gel in 25 % isopropyl alcohol followed by 10 % acetic acid for overnight at room temperature then soak in 7.5 % acetic acid for 30 min. Transfer the gel in 0.2 % aqueous periodic acid for 1 h at 4 °C. Wash the gel with several changes of distilled water for 3 h. Treat gel with freshly prepared, filtered ammoniacal silver solution for 10 min. Immediately wash for 2 min in distilled water. Transfer the gel into freshly prepared solution containing 0.005 % citric acid, 0.019 % formaldehyde solution, and 10 % methanol. Wash the gel thoroughly in distilled water for 1 h with agitation and several changes.

3.7 Expression of Haa86 in Prokaryotic Expression System (E. coli)

1. Amplification of the targeted sequence: For 50 μl reaction add 5.0 μl 10× Taq buffer, 1.0 μl 10 mM dNTPs, 2 μl each of 10 mM forward (HA3) and reverse (HA4) primer, 1 μl (10–100 ng) Haa86 positive plasmid (pTZHA86), and 0.4 μl Hot Start Taq Polymerase (5 unit/μl). Run the PCR as initial denaturation at 95 °C for 5 min and further 30 cycles at 94 °C for 1 min, 47 °C for 1 min, and 68 °C for 2 min and a final extension at 68 °C for 10 min.
2. Extraction of amplified product: Resolve the PCR product in 1 % agarose gel along with 100 bp plus DNA ladder and slice out the gel region containing targeted amplicons. Isolate the amplicons from gel slice following the protocol and reagent of gel extraction kit.
3. Quantify the amplicons concentration using NanoDrop spectrophotometer.
4. Digestion of the amplicons and expression vector (pET32a): Set up the reaction separately for amplicons and vector. For 20 μl reaction, use 2 μl vector (1 μg), 2 μl 10× RE buffer, 1 μl each of EcoRI and XhoI restriction enzymes, and 14 μl of NFW. Incubate at 37 °C for 2 h followed by 70 °C for 10 min. Similarly, use 0.2 μg of amplicons in reaction and follow the above guide lines. Purify both digested vector and amplicons using gel extraction kit.
5. Quantify the concentration of digested vector and amplicons.
6. Setting up of ligation reaction: Typically for 20 μl reaction, add 2 μl gel-purified digested vector (100 ng) and 5 μl gel-purified digested amplicons (250 ng) in a 0.2 ml PCR tube and incu-

bate at 65 °C for 5 min, then add 4 μl 5× buffer, 1 μl T4 DNA ligase, and 8 μl nuclease-free water. The ligation is incubated at 4 °C overnight and then heat killed at 70 °C for 10 min.

7. Transformation of ligated product into *E. coli* NovaBlue® competent cells: Add 2 μl of ligation mix into 0.5 ml tube containing pre-aliquoted 20 μl competent cell on ice. Incubate for 5 min on ice then give heat shock (immerse the tube for 45 s in water with a temperature of 42 °C and again put on ice). Incubate for 5 min on ice. Add 100 μl SOC media in the above tube at room temperature and incubate at 37 °C for 45 min. Spread the transformed cell suspension on pre-warmed (37 °C) LB agar plate containing ampicillin using sterile spreader under laminar air flow. After drying of surface moisture, wrap the plate in aluminum foil and incubate at 37 °C upside down in a bacteriological incubator for overnight.
8. Select the bacterial colony from the plate having circular circumference.
9. Confirmation of the positive colony by colony PCR: Take small part of colony in a 50 μl NFW using bacteriological loop or 10 μl tip in a 0.5 ml tube. Boil for 10 min in boiling water bath and centrifuge at 10,000 g for 2 min. Use the supernatant as a template DNA in a PCR reaction as stated above (*see* Subheading 3.7, **step 1**).
10. Growing of the PCR confirmed colony in LB broth containing 100 μg/ml ampicillin: Leftover colony after colony PCR mix in a 5 ml LB broth containing 100 μg/ml ampicillin present in 50 ml sterile tube. Incubate at 37 °C in a shaking incubator for 5–6 h.
11. Use QIAprep Spin Miniprep Kit for isolation of bacterial plasmid (pETHA86).
12. Testing for insert release: Set up the 20 μl plasmid digestion reaction. Use the vector digestion protocol (*see* Subheading 3.7, **step 4**).
13. Transformation of positive plasmid (pETHA86) into expression host *E. coli* BL21(DE3)PLysS (*see* Subheading 3.7, **step 7**). Use LB agar plate containing both ampicillin and chloramphenicol.
14. Growing of 5–6 bacterial colonies in LB broth containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol: Number the colonies using marker pen on plate. Take out half the colony from plate and add in 5 ml LB broth containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol present in 50 ml sterile tube. Incubate it at 37 °C in a shaking incubator for 3–4 h. Induce the culture by adding 5 μl of 1 M IPTG and grow additionally for 3–4 h. Keep one tube as uninduced, i.e., without IPTG.
15. Selection of the bacterial colonies having good expression: Collect 1 ml culture from each tube into labeled 1.5 ml

Eppendorf tube. Centrifuge at 12,000 × *g* for 1 min. Discard the supernatant and dissolve the bacterial pellet into 50 μl distilled water. Add 10 μl 5× sample buffer and mix well. Boil the content present in 1.5 ml tube for 10 min in boiling water bath. Centrifuge at 12,000 × *g* for 2 min. Load 10 μl of this cell lysate in 10 % polyacrylamide gel containing SDS along with protein marker. Run the PAGE to resolve the proteins. After 20 min of staining into Coomassie Brilliant Blue R250 stain, destain the gel in destaining solution for 1–2 h. Observe the band of expressed protein in gel at expected molecular weight.

16. Preparation of glycerol stock of good expressed bacterial clone(s): Review the recorded data to identify clones having good expression by retrospective study of data recorded in data book. Grow the colony present in plate into 5 ml LB broth containing antibiotics as above for 4–5 h. Subculture in 2 ml LB broth containing antibiotics in 15 ml sterile tube. Add 200 μl of bacterial culture and grow at 37 °C in a shaker incubator for 1 h. Place the tube on ice and add 330 μl of pure sterile glycerol and mix it. Make the 0.5 ml aliquot in sterile 1.5 ml tubes. First freeze at −20 °C for 2–4 h and finally store at −80 °C.

3.8 Purification of Recombinant Protein

1. Preparation of fresh culture of positive clone: Thaw a glycerol stock of clone on ice and centrifuge at 10,000 × *g* for 1 min to pellet the bacteria. Discard the supernatant and add the pellet into 5 ml LB broth containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol present in 50 ml sterile tube. Incubate in shaker incubator (37 °C and 150 rpm) for 5 h. Again subculture it in 5 ml LB broth with antibiotics as stated above, overnight.
2. Shake flask culture of bacterial clones: Autoclave 50 ml LB broth in a 250 ml flask, cool to 37 °C, and add 100 μg/ml ampicillin, 34 μg/ml chloramphenicol, and 500 μl overnight cultured bacteria. Incubate in shaker incubator (37 °C and 150 rpm) for 3–4 h (till OD_{600} reached 0.5–1.0). Induce the culture by adding 50 μl of 1 M IPTG for the expression of protein in next 5 h at 37 °C.
3. Collection of bacterial lysate: Pellet the cultured bacteria by centrifuging at 10,000 × *g* for 5 min. Discard the supernatant and record the weight of the pellet by subtracting the weight of empty centrifuge tube. Add 2 ml lysis buffer (5 ml/g of pallet) and mix properly. Incubate the mixture at 4 °C for 1 h and sonicate at 10,000 Hz for 30 s, 4–5 times with an interval of 60 s on ice. Centrifuge the content at 25,000 × *g* for 30 min at 4 °C. Collect the clear lysate in a fresh tube and store at −20 °C.
4. Equilibration of the Ni-NTA superflow resin: Load the 1 ml Ni-NTA resin in column. Open the lower vent to remove the preservative. Subsequently, pass the 10 ml lysis buffer pH 8.0 through column. Close the lower vent before Ni-NTA beads are directly exposed to air.

5. Binding of protein with Ni-NTA: Mix the equilibrated Ni-NTA resin to lysate at room temperature and place on rocker for 30 min.
6. Elution of purified protein: Reload the Ni-NTA-lysate mix into column and open the lower vent. Wash the resin in column by flowing 10 ml each of wash buffer, pH 6.3, and wash buffer, pH 5.9, through the column. Finally, add the elution buffer pH 4.5. Collect the flow through in 1.5 ml tubes as 0.5 ml fraction up to 6–7 fractions.
7. Resolving eluted fractions in 10 % SDS-PAGE: Prepare the 10 % gel and load 10 μl eluted protein along with 5× sample buffer (10 μl protein + 2 μl sample buffer) after boiling for 10 min at water bath. In central well, load 8 μl protein molecular weight marker. Stain and destain the gel and see the resolved protein bands using gel documentation and imaging system.
8. Removal of urea from the eluted fractions: Pool the fraction 2–6 (having high concentration of protein shown on SDS-PAGE) in 10 kDa molecular weight cutoff (MWCO) dialysis bag and dialyzed against descending order of urea concentration: 6, 4, and 2 M and finally in PBS pH 7.2 for 8–12 h at each concentration.
9. Concentration of the dialyzed protein: Collect the finally dialyzed protein from dialysis bag in a molecular weight cutoff filter, centrifuge at 5000 × *g* for 50–60 min at 4 °C. Measure the concentration of protein using spectrophotometer and add 10 μl cocktail protease inhibitor in 1 ml of the final concentrated protein.
10. Properly label the tube and store the protein at 4 °C for short period and −20 °C for longer periods.

4 Notes

1. Healthy New Zealand white rabbits of 9 months to 1 year old and 1.5–2 kg in weight are to be used for feeding of larvae of *H. anatolicum*. After feeding, the engorged larvae will remain on rabbits to molt into unfed nymphs, which then attach and engorge. The engorged nymphs are to be collected and cleaned before placing in tick rearing glass vials. The tubes containing engorged nymphs are to be kept at 28 °C and in 85 % RH for molting. The freshly hatched adults were kept unfed for 7 days. The adults are to be released on more than 6-month-old crossbred calves. The ear bags need to be checked daily, for collection of the fed adults. Collected adults are to be cleaned, weighed, labeled, and kept alone in the glass tubes at 28 °C and 85 % RH for oviposition [13].
2. Ticks should be cleaned in a cleaning solution to remove any surface-attached dirt and microbes.

3. DEPC (Diethylpyrocarbonate) used to inactivate and destroy the RNase. RNase is ubiquitously present everywhere in environment. To safeguard the extracted RNA from RNase, all the equipments and reagents should be free from RNase.
4. Hard exoskeleton of ticks becomes fragile when incubated in liquid N_2. It helps to break the tissue into fine particles to improve the total RNA extraction.
5. RNaseZap®, a commercial product, contains the chemicals which inhibits the RNase ubiquitously present in environment. Apply this product on work surface and hand gloves to minimize the RNase contamination.
6. TRIzol® is a commercial product that contains phenol, guanidine isothiocyanate, and other proprietary components. It has very effective control over the RNase released from breaking cells and tissues [23]. This reagent is very effective for the isolation of high-quality total RNA from ticks.
7. Chloroform denatures the contaminated protein and converts soluble into organic phase.
8. Isopropyl alcohol precipitate the RNA. Overnight incubation at –20 °C gives better yields of RNA than 4 °C for 10 min.
9. Washing of RNA pellet is very essential to remove any contaminating chemicals present in it. RNA does not dissolve in 70 % ethanol but provide the environment to leach out the contaminating chemicals from RNA pellet.
10. Ethanol acts as an inhibitor in many downstream processing (cDNA preparation, PCR reaction) of RNA. Care should be taken to avoid RNA pellet over drying during ethanol evaporation. Before drying, add nuclease-free water in RNA pellet.
11. Based upon the conserved region of ticks gene available in GenBank (NCBI), either degenerate or normal primer is designed with the help of bioinformatics software like GeneTool, DNAstar, etc., which gives maximum length of nucleotide sequence of interest.
12. Ligation can be performed with different incubation temperature and time combinations. Here pre-incubation of vector and insert at 65 °C for 5 min significantly improves the ligation efficiency.
13. Cloning kit provides reagents for the preparation of competent cells. Alternatively, laboratory procedure can be followed. Grow *E. coli* DH5α overnight in shaker incubator (37 °C, 120 rpm). One milliliter of this culture is to be seeded in 50 ml of autoclaved LB media and grow for 3 h at 37 °C, 120 rpm.

Harvest the cells by centrifuging at 7000×*g*/4 °C for 2 min. Suspend the cells in 17 ml sterile 0.1 M $CaCl_2$ (one third volume of the bacterial culture) and incubate on ice for 60 min. Pellet the cells and resuspend in 2 ml of solution containing 1.7 ml of 0.1 M $CaCl_2$ and 0.3 ml of autoclaved glycerol (15 % glycerol). Aliquot into 200 µl and store at −80 °C.

14. For the transformation in competent cells, follow this protocol: After transferring the 5 µl of ligation mix into thawed competent cells, incubate on ice for 5 min, then give heat shock by short incubation at 42 °C for 45 s, and immediately keep on ice for 30 min. Add 200 µl of freshly autoclaved LB broth and incubate at 37 °C for 45 min in a shaker incubator. Use 200 µl to spread on agar plate and the rest is stored at 4 °C.
15. For blue-white screening of recombinant clones, the design vector (pTZ57R/T cloning vector) has a short fragment of *lacZ* gene called α-fragment (*lacZα*), whereas mutated host cells (like *E. coli* strain like DH5α) has *lacZ* gene minus α-fragment (*lacZα*) called ω-fragment (*lacZΔM15*) of β-galactosidase gene (*lacZ*). When the product of α-fragment combines with product of ω-fragment (called α-complementation), functional β-galactosidase is formed. The *lacZα* fragment in vector contains multiple cloning sites (MCS) for inserting the targeted gene. When MCS are free of insert sequence, uninterrupted *lacZα* produces α-fragment and functional β-galactosidase is formed which cleaves the X-gal (added in media), a colorless analogue of lactose, to form 5-bromo-4-chloro-indoxyl which gives the blue color, whereas when insert is present in MCS, the *lacZα* is altered leading to absence of α-complementation and cleavage of X-gal, resulting in the absence of blue color development. Select only white, good size, lonely bacterial colony with circular circumference. Do not select the bacterial colony which is closely surrounded by small bacterial colonies.
16. Linearization of the recombinant *Pichia* vector is required for homologous recombination of the 5′ *AOX1* and 3′ *AOX1* with the *P. pastoris* genome and subsequent integration of the foreign gene into the *P. pastoris* genome.
17. The *P. pastoris* GS115 (*his4*) is a mutant methylotrophic yeast strain which cannot synthesize histidine de novo. The gene encoding histidinol dehydrogenase, involved in the histidine synthesis pathway, was disrupted in this strain.

Acknowledgements

Sincere thanks are due to Indian Council of Agricultural Research and Department of Biotechnology, Government of India, for providing funds to establish different protocols in the laboratory.

References

1. Ghosh S, Bansal GC, Gupta SC, Ray DD, Khan MQ, Irshad H, M S, Seitzer U, Ahmad JS (2007) Status of tick distribution in Bangladesh, India and Pakistan. Parasitol Res 2:S207–S216
2. de la Fuente J, Almazan C, Canales M, de la Lastra JM, Kocan KM, Willadsen P (2007) A ten-year review of commercial vaccine performance for control of tick infestations on cattle. Anim Health Res Rev 8:23–28
3. Willadsen P, Bird P, Cobon GS, Hungerford J (1995) Commercialization of a recombinant vaccine against *Boophilus microplus*. Parasitology 110:S43–S50
4. Canales M, Enriquez A, Ramos E, Cabrera D, Dandie H, Soto A, Falcon V, Rodriguez M, de la Fuente J (1997) Large scale production in *Pichia pastoris* of the recombinant vaccine Gavac™ against cattle tick. Vaccine 15:414–422
5. Willadsen P, Kemp DH (1988) Vaccination with 'concealed' antigens for tick control. Parasitol Today 4:196–198
6. Kumar B, Azhahianambi P, Ray DD, Chaudhuri P, de la Fuente J, Kumar R, Ghosh S (2012) Comparative efficacy of rHaa86 and rBm86 against *Hyalomma anatolicum anatolicum* and *Rhipicephalus* (*Boophilus*) *microplus*. Parasite Immunol 34:297–301
7. Azhahianambi P, de La Fuente J, Suryanarayana VV, Ghosh S (2009) Cloning, expression and immunoprotective efficacy of rHaa86, the homologue of the Bm86 tick vaccine antigen, from *Hyalomma anatolicum anatolicum*. Parasite Immunol 31:111–122
8. Eukart MR, Bussineau CM (1996) Quality and authenticity of heterologous proteins synthesized in yeast. Curr Opin Biotechnol 7:525–530
9. Hollenberg CP, Gellissen G (1997) Production of recombinant proteins by methylotrophic yeasts. Curr Opin Biotechnol 8:554–560
10. Sorensen HP, Mortensen KK (2005) Advanced genetic strategies for recombinant protein expression in *Escherichia coli*. J Biotechnol 115:113–128
11. Jongejan F, Uilenberg G (2004) The global importance of ticks. Parasitology 129:S3–S14
12. Ghosh S, Nagar G (2014) Problem of ticks and tick-borne diseases in India with special emphasis on progress in tick control research: a review. J Vector Borne Dis 51:259–270
13. Ghosh S, Azhahianambi P (2007) Laboratory rearing of *Theileria annulata*-free *Hyalomma anatolicum anatolicum* ticks. Exp Appl Acarol 43:137–146
14. Shyma KP, Kumar S, Sangwan AK, Sharma AK, Nagar G, Ray DD, Ghosh S (2013) Acaricide resistance status of *Rhipicephalus* (*Boophilus*) *microplus* and *Hyalomma anatolicum* collected from Haryana. Indian J Anim Sci 83:591–594
15. Celik E, Calik P (2012) Production of recombinant proteins by yeast cells. Biotechnol Adv 30:1108–1118
16. Balamurugan V, Reddy GR, Suryanarayana VVS (2007) *Pichia pastoris*: a notable heterologous expression system for the production of foreign proteins—Vaccines. Indian J Biotechnol 6:175–186
17. Rosano GL, Ceccarelli EA (2014) Recombinant protein expression in *Escherichia coli*: advances and challenges. Front Microbiol 5:172
18. Studier FW, Moffatt BA (1986) Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J Mol Biol 189:113–130
19. Rosenberg AH, Lade BN, Chui DS, Lin SW, Dunn JJ, Studier FW (1987) Vectors for selective expression of cloned DNAs by T7 RNA polymerase. Gene 56:125–135
20. Studier FW, Rosenberg AH, Dunn JJ, Dubendorff JW (1990) Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol 185:60–89
21. Stano NM, Patel SS (2004) T7 lysozyme represses T7 RNA polymerase transcription by destabilizing the open complex during initiation. J Biol Chem 279:16136–16143
22. Novy R, Morris B (2001) Use of glucose to control basal expression in the pET system innovations. BioTechniques 13:8–10
23. Rio DC, Ares M Jr, Hannon GJ, Nilsen TW (2010) Purification of RNA using TRIzol (TRI reagent). Cold Spring Harb Protoc pdb. prot5439