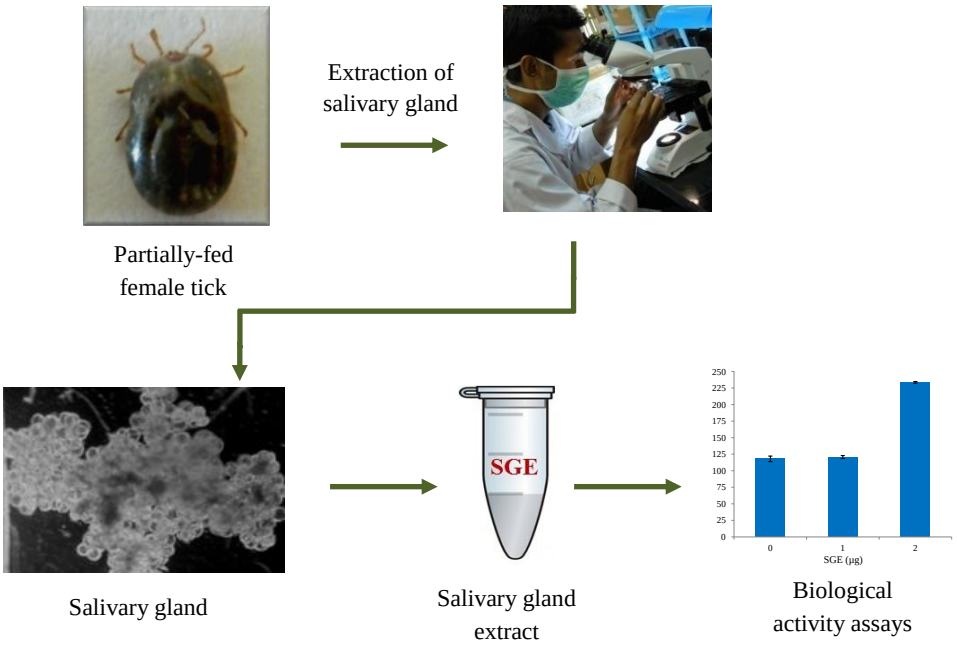
Chapter 3
Extraction and characterization of salivary gland extract

Graphical abstract



3.1 Introduction

Ticks are haematophagus organism which feed on terrestrial vertebrates, including amphibians, reptiles, birds and mammals. For continuous feeding they inject haemostatically active protein into the host which is produced and stored in their salivary glands. In the previous chapter, we have identified the ticks infesting cattle, in Napaam village, Sonitpur, India as *Rhipicephalus* (*Boophilus*) *microplus* and *Haemaphysalis bispinosa*. In this chapter, the extraction of the salivary glands from the identified ticks was undertaken. Preliminary investigation of the salivary gland

extract (SGE) for anti-coagulation property was done to assess its ability to inhibit blood coagulation *in vitro*. The SGE was tested for inhibition of plasma clotting by determining the recalcification time, prothrombin time and activated partial thromboplastin time. SGE delayed clotting time of platelet poor plasma significantly which indicates that it possesses anti-coagulant proteins or polypeptides.

3.2 Materials and methods

3.2.1 Materials

Partially-fed female *H. bispinosa* ticks were collected from Napaam village, Assam (Latitude 26.70420^0 N and Longitude 92.83340^0 E). Pierce™ Silver staining kit was from Thermo Scientific (Rockford, USA). Uniplastin and Liquecelin were from Tulip Diagnostics (P) Ltd. (Verna, India). All other chemicals and reagents used were of analytical grade and purchased from Sigma (MO, USA), Merck (Mumbai, India) and Himedia (Mumbai, India).

3.2.2 Isolation of Salivary Glands (SG)

For the isolation of the salivary glands the method of Edwards *et. al.* [251] was followed with some modification. Partially-fed female tick was placed onto a petri-dish and the scutum was removed using a sterile blade under a stereo-microscope. The blood meal was washed off with 20 mMTris-Cl, 150 mM NaCl (pH 7.4) and the salivary gland was dissected out using forceps. The extracted glands were washed in the same buffer and unwanted tissues removed carefully and stored at -80°C until use.

3.2.3 Preparation of Salivary Gland Extract (SGE)

Salivary gland extract was prepared according to Mans *et. al.* [112], with some modification. Salivary glands from about 100 adult female ticks were reconstituted in 20 mM Tris-Cl, pH 7.4 and homogenized in ice using IKA Tissue Homogenizer (3000 rpm, 2-3 min). The lysate was centrifuged at 12,000 rpm for 10 min at 4°C. The clarified supernatant was collected which constituted the SGE and stored at -20°C till further use.

3.2.4 Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was carried out according to Laemmli [252] with some modifications. Briefly, for 12.5% resolving gel, 8.3 ml of Acrylamide/bisacrylamide (29:1) was added to a final volume of 20 ml containing 0.1% SDS, 4% glycerol, 5 ml 1.5 M Tris-Cl (pH 8.8), 0.1% Ammonium persulfate (APS) and 20µl N, N, N', N'-tetramethylethylenediamine (TEMED). 5% stacking gel was prepared by mixing 0.65 ml 30% Acrylamide/bisacrylamide, 0.1% SDS, 4% glycerol, 1.25 ml 0.5 M Tris-Cl (pH 6.8), 0.1% APS and 7.5 µl TEMED to a final volume of 5 ml. The samples were prepared by adding sample buffer (2% SDS, 10% glycerol, 0.2% Bromophenol blue, 0.25 M Tris-Cl, pH 6.8) containing 3% 2-mercaptoethanol and heated for 2 min in boiling water. After electrophoresis at constant current of 20-30 mA in Tris-Glycine-SDS buffer (25 mM Tris, 192 mM Glycine, 0.1% SDS), the gel was silver-stained. The gel was photographed and documented.

3.2.5 Silver staining of SDS-PAGE gel

After electrophoresis, the gel was transferred from the electrophoretic cassette to a clean container and stained with silver stain. Briefly, the gel was washed for 2 min in ultrapure water for two times. The gel was fixed in ethanol, acetic acid and water (30:10:60) solution for 1 h, followed by washing twice in 10% ethanol for 2 min each, then twice in ultrapure water for 2 min each. The gel was then sensitized in the sensitizer solution (50 µl Sensitizer solution with 25 ml water) for 1 min and washed with water twice for 1 min each. The gel was stained with the staining solution (0.5 ml Enhancer with 25 ml Stain) for 30 min, washed twice with ultrapure water for 20 s each and developed for 2-3 min in developer solution (5 ml Enhancer with 25 ml Developer) until bands appeared. The reaction was stopped with 5% acetic acid for 10 min.

3.2.6 Preparation of platelet poor plasma from citrated goat plasma

For the preparation of platelet poor plasma (PPP), citrated goat blood was centrifuged twice at 2500 g for 20 min at room temperature. The PPP was then collected in 2 ml aliquots and stored at -20°C till further use [253]. The plasma was used within 4 h after thawing.

3.2.7 Coagulation assays

Recalcification time assay was done according to Doley and Mukherjee (2003) [253], with some modification. 100 µl of citrated goat plasma was incubated with/without SGE and 50 µl of Tris buffer (pH 7.4) at 37°C for 2 min. Coagulation was triggered by adding 100 µl of 100 mM $CaCl_2$. Prothrombin time (PT) and activated partial thromboplastin time (APTT) were performed by pre-incubating citrated goat plasma (50 µl) with different concentrations of the SGE (50 µl) or 20 mM Tris-Cl pH 7.4, 100 mM NaCl at 37°C for 2 min, followed by addition of corresponding reagents (PT: 50 µl of Uniplastin; APPT: 50 µl of Liquecelin added for 3 min and reaction started with 50 µl of 20 mM $CaCl_2$) [116]. The time of clot formation was recorded using a COAstat-1 coagulation analyzer (Tulip groups, Verna, India).

3.2.8 Hemolytic activity assay

Hemolytic activity of SGE was evaluated according to Tambourgi *et. al.* [254], with slight modifications. SGE/water was added to 5% RBC and volume was adjusted to 2 ml by adding 0.9% NaCl. The mixture was incubated at 37°C for 1 h and centrifuged at 5000 rpm for 10 min. Supernatant was collected and absorbance was recorded at 540 nm. The percentage hemolytic activity was calculated taking hemolysis by water as 100%.

3.2.9 PLA_2 activity assay

PLA_2 activity of SGE was assayed by well-diffusion method [255]. 8% egg yolk was mixed with molten agar medium after cooling to about 45°C. The mixture was poured onto 90 mm petri-plate and allowed to solidify. Wells were punched using a sterilized borer and required amount of SGE/150 mM NaCl was added into the wells and incubated at 37°C for 3-5 h. 5µg of crude *Bungarus fasciatus* venom was used as positive control.

3.3 Results

3.3.1 Isolation of salivary gland and preparation of Salivary Gland Extract

The salivary glands from the fully fed ticks were successfully dissected out under a microscope (Fig. 3.1). The glands appeared as clear, grape-like structures. A pair of glands was extracted from each tick specimen.

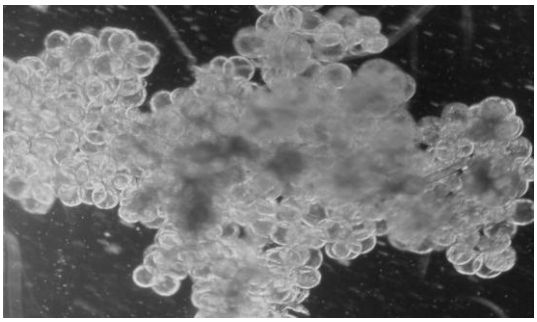


Figure 3.1 Representative of a salivary gland dissected out from *H. bispinosa* female tick (10x magnification).

3.3.2 SDS-PAGE gel analysis of Salivary Gland Extract

Homogenized and clarified lysate of salivary glands, which constituted the SGE, was run on 12.5% SDS-PAGE gel (Fig.3.2). Abundance of a large number of proteins ranging from 25 kDa to 250 kDa was observed on the gel. Bands of size ranging from 10-20 kDa were also observed on the gel, which represent the size of most of the anti-coagulant proteins, mainly thrombin inhibitors, isolated from ticks.

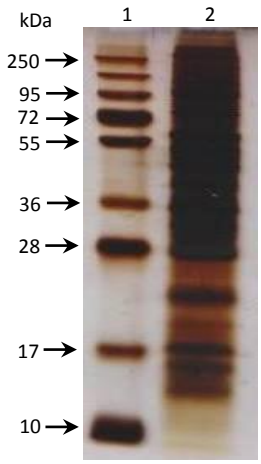


Figure 3.2 SDS-PAGE gel profile of salivary gland extract. Lane 1: Protein molecular weight marker; lane 2: *H. bispinosa* salivary gland extract.

3.3.3 Recalcification time of SGE

SGE delayed clot time of the platelet poor plasma. The recalcification time of platelet poor plasma was found to be 118 ± 4.24 s. However, the same was prolonged to 233.55 ± 1.34 s when PPP was incubated with 8 µg ml^{-1} of SGE (Fig. 3.3).

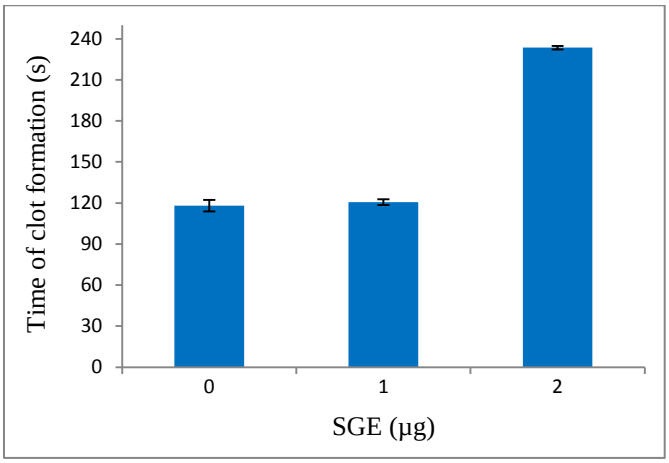


Figure 3.3 Recalcification time of PP plasma when pre-incubated with SGE (Salivary Gland Extract) of *H. bispinosa* (n = 3, error bars represent ±S.D.).

3.3.4 Activated partial thromboplastin time (APTT) of SGE

Salivary gland extract of *H. bispinosa* delayed APTT of platelet poor plasma (Fig. 3.4). The APPT of PPP was recorded to be 31.96 ± 0.47 s, while that for PPP treated with 8 µg ml^{-1} of SGE was found to be 86.45 ± 6.15 s, which is about 2.7 fold the normal clotting time.

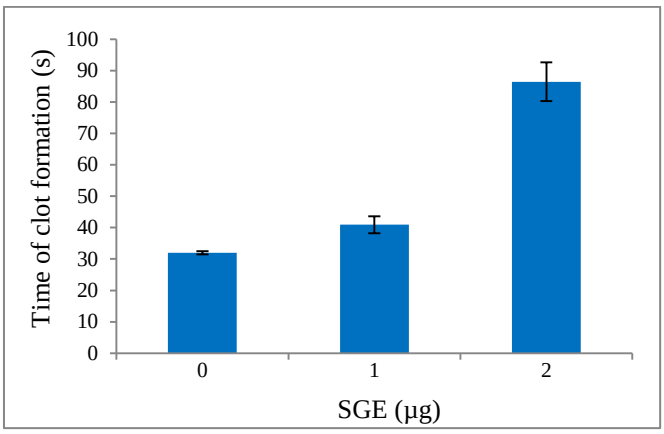


Figure 3.4 Activated partial thromboplastin time of PP plasma pre-incubated with SGE (Salivary Gland Extract) of *H. bispinosa* (n = 3, error bars represent ±S.D.).

3.3.5 Prothrombin time (PT) of SGE

Salivary gland extract of *H. bispinosa* did not delay PT of platelet poor plasma (Fig. 3.5). There was no visible change in the PT of PPP, when it was incubated with 8 µg ml^{-1} of SGE.

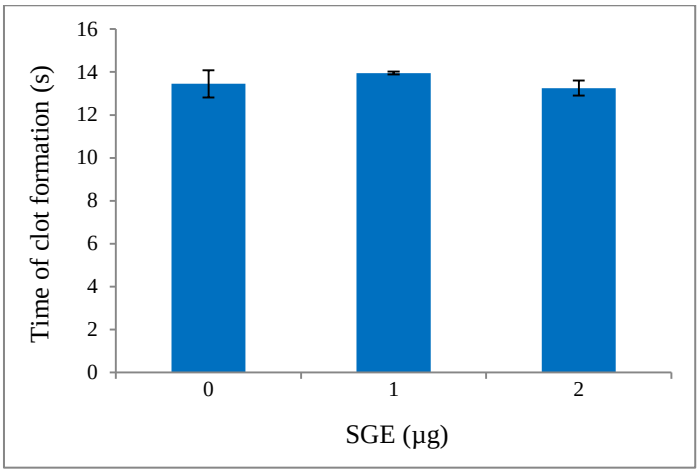


Figure 3.5 Prothrombin time of PP plasma pre-incubated with SGE (Salivary Gland Extract) of *H. bispinosa* (n = 3, error bars represent ±S.D.).

3.3.6 Hemolytic activity of SGE

Salivary gland extract of *H. bispinosa* did not show any hemolysis of RBC (Fig. 3.6), suggesting that it does not have any membrane damaging property. The lysis of RBC (positive control) in the presence of water was taken as 100%.

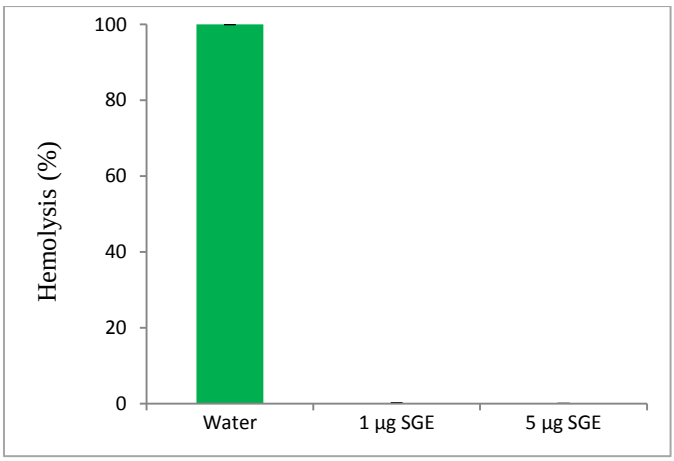


Figure 3.6 Percentage hemolysis of RBC by SGE (Salivary Gland Extract) of *H. bispinosa*. Percent hemolysis was calculated considering the hemolysis by water as 100% (n = 3, error bars represent ±S.D.).

3.3.7 PLA_2 activity of SGE

Varying amount of SGE was added to the wells on egg-yolk agar plate and the medium was observed for clearance of zone. No zone of clearance was seen in any wells loaded with SGE, showing that it did not show any PLA_2 activity (Fig. 3.7). A clear zone was observed in the well treated with *Bungarus fasciatus* venom (positive control); *B. fasciatus* venom is known to have PLA_2 activity.

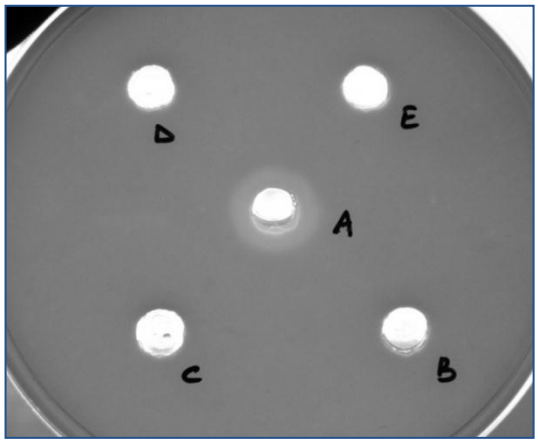


Figure 3.7 PLA_2 activity of SGE of *H. bispinosa*. Well A: 5 µg *Bungarus fasciatus* venom; well B: 150 mM NaCl; well C: 1 µg SGE; well D: 2 µg SGE; well E: 5 µg SGE.

3.4 Discussions

For successful feeding, ticks evade the host hemostatic response by injecting saliva, which consist of vast range of pharmacologically active molecules which are produced and stored in the salivary gland [101,104-106]. Saliva from the same tick may contain many anti-hemostatic molecules that target the coagulation cascade or sometimes a single molecule might target various factors. These biologically active molecules include immunomodulating substances, vasodilators, anticoagulants, platelet adhesion and aggregation inhibitors and fibrin(ogen)olytic agents [170]. Therefore, study of the protein profile of the tick salivary gland from the stand point of biochemistry, pharmacology and molecular biology will help us to understand the various proteins present in the salivary gland which are involved in interacting with the host hemostatic system. These proteins can be explored for potential anticoagulant drug lead, as these target specific clotting factors of the coagulation cascade. They can

also be used for therapeutic applications such as treatment of thromboembolic diseases. In this chapter, we have characterized the salivary gland extract of *Haemaphysalis bispinosa* for its anti-coagulation properties.

Ticks have evolved in a course of few hundred million years to feed on host blood, and are good source of anti-coagulant proteases [170,256]. Salivary gland extract of ticks is a potent source of anti-hemostatic proteins, particularly thrombin inhibitors [116]. Koh *et. al.* (2007) reported that the salivary gland extract from *Amblyomma variegatum* showed potent anti-coagulation property when it was tested for APTT, PT and thrombin time (TT), in the order TT >> APTT > PT [116]. They purified and characterized a novel thrombin inhibitor called Variegin from its saliva which binds active site and exosite-I of the thrombin [116]. Salivary gland extract of soft tick, *Argas monolakensis* prolonged APTT and PT of human plasma significantly and the prolongation of recalcification time of chicken plasma was also reported [112]. Thrombin inhibitor, monobin and platelet aggregation inhibitors, monogrins which are orthologous to those isolated from genus *Ornithodoros*, were described from the salivary gland of *A. monolakensis* [112,146,147]. Several other non-orthologous thrombin inhibitors which include boophilin, madanins, amblin, microphilin and chimadanin have been identified in hard ticks [109,118,195,224,257,258].

Haemaphysalis bispinosa is a cattle tick which is prevalent in this region. Salivary glands were isolated from *H. bispinosa* adult female ticks by dissection under microscope and Salivary Gland Extract (SGE) was then prepared by homogenization. SGE is a good alternative to saliva, since it is easier to collect and it possesses the attributes like that of saliva [259]. Though SGE contains cellular proteins besides the salivary contents, it has the advantage that it is free from pilocarpine, which is commonly used to induce salivation when collecting saliva from ticks. Pilocarpine is a muscarinic cholinomimetic agent that induces salivation and has been shown to be cytotoxic to *B. burgdorferi* during saliva collection [260,261], a certain disadvantage to researcher studying pathogens transmitted by ticks. In the present study, our main objective was isolation of anti-coagulant protein form salivary glands of the ticks. Therefore, our primary concern was preparing gland extract absolute of any tissue contamination.

To examine the potency of SGE to inhibit coagulation of platelet poor plasma (PPP), it was tested for Recalcification time, Activated Partial Thromboplastin time and Prothrombin time. The Recalcification time of PPP was prolonged to ~2 fold by SGE and APPT was prolonged to ~2.7 fold. However, unlike SGE of *A. variegatum*, SGE of *H. bispinosa* did not inhibit the Prothrombin time of PPP, which is a test for the extrinsic pathway of the coagulation cascade. Thus, it is apparent that the isolated tissue is indeed the salivary gland of *H. bispinosa* and it affects the intrinsic pathway of the coagulation cascade (APTT test), but not extrinsic pathway of the cascade. For continuous feeding, tick inserts its hypostome into the host skin injuring its epidermis and injects saliva which contains myriad of anti-haemostatic proteins, anti-inflammatory and immunomodulatory substances. Though ticks injure the host's epidermal layer, it requires that the host be alive and it caused no harm to the host tissue. Therefore, its saliva should not contain any substances which may damage the host tissues. When SGE was tested for hemolysis (break down of RBC) and PLA_2 activity (phospholipid hydrolyzing enzyme) [262], it did not show any effect, suggesting that the proteins and polypeptides present in the saliva does not have any membrane damaging property.

When SGE was analyzed on SDS-PAGE gel, bands of proteins ranging from about 10 to 250 kDa were observed. Majority of which were found at the range of 30-250 kDa and about 17-20 kDa, which includes the cellular proteins besides the proteins expressed in the salivary glands. After successfully attachment to the host skin at the wound site, ticks feed on the host blood for several days [76]. The different stages of feeding includes (i) attachment onto host skin, installing a feeding lesion; (ii) slow feeding and preparing itself for the next stage by growing tissue; transmits pathogens in the process (iii) rapid feeding till repletion [76,83]. During the course of these three stages, the tick salivary gland produces/expresses different types of proteins to aid the feeding process. Tirloni *et. al.* identified several proteins families in the saliva of partially and fully engorged *R. microplus* ticks by proteomic approach which included lipocalins (8-26 kDa), secreted conserved proteins (6-52 kDa), serpin (44.4 kDa), TIL domain-containing proteins (9-18 kDa), thyropin (20-30 kDa), peptidases like tripsin-like, metalloproteases, cathepsin B-like (38-59 kDa), phospholipases (40-70 kDa), glycine-rich proteins (9-67 kDa), Antigen 5 (45.9 kDa),

transporter proteins, ferritin 1 (21.4 kDa) and vitellogenin (201.8 kDa), calreticulin 1 (47.8 kDa), kazal domain-containing (32.7 kDa), kunitz domain-containing (78.2 kDa), house-keeping proteins (6-84 kDa), immunoglobin G-binding protein (17-20 kDa), cysteine-rich KGD motif-containing protein 1 (19 kDa), and histidine-rich antimicrobial peptide (17.5 kDa) [263]. Presence of several protein bands in the range of 10-250 kDa in the SDS-PAGE profile of SGE *of H. bispinosa* might be the presence of these proteins or its homologues (described above). Literature survey shows that none of these proteins from the SGE of *H. bispinosa* prevalent in this region has been reported so far. As discussed earlier, tick anti-hemostatic proteins are potential target for drug discovery because of their specificity. Besides this, salivary components can be targeted for development of anti-tick vaccines, as ticks and tick-borne diseases pose global threat for livestock and human health [264]. The current control of ticks based on chemical acaricides lays serious health and environmental hazards, apart from development of acaricide resistance in ticks and deposition of chemical residues in milk and meat [265,266]. Therefore, tick vaccines based on tick secretory and cellular proteins are the choice of control, since the development of antigen based vaccine Bm86, an antigen from *R. microplus* midgut [267]. Several secreted, membrane associated and intracellular tick antigens have been isolated and tested in animal trials, few of which are Ras-3, Ras-4 (serine protease inhibitors from *R. appendiculatus*) [195], BmLTI, BmTI, BmTI-A (tripsin inhibitors from *R. microplus*) [189,268,269], GP80/VIT87 (Vitellin/Vitellogenin from *R. microplus*) [270], Voraxinα (Tick mating factor from *R. appendiculatus*) [271], pP0 (acidic ribosomal protein P0 from *R. microplus*) [272], Subolesin (regulator factor from *R. annulatus* and *R. microplus*) [273], Aquoporin (Aquoporin from *R. microplus* and *R. annulatus*) [274] and so on. Therefore, studying tick secretory, midgut and hemolymph proteins can lead to identification of potential vaccine candidates that can be used for control of ticks and tick borne diseases. Additionally, novel molecules, mainly anti-hemostatic proteins may be discovered from the saliva which can be prospective drug candidate against hemostatic disorders and cardiovascular diseases.