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Characterization of protein profile and evaluation of immune-peptide of crude and Excretory/Secretory antigens of some haematophagus leeches of India

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ABSTRACT

Leech infestation leads to significant blood loss compared to other parasites, causes irritation in humans and predisposes livestock to secondary fly attack and maggot infestation. During blood feeding, leeches inject saliva containing bioactive proteins at the bite site that facilitate continuous blood flow and provoke inflammatory responses. The present study was undertaken to characterize the crude and excretory/secretory (E/S) antigens of three common leech species of Northeast India- two aquatic (*Hirudinaria manillensis* and *H. granulosa*) and one terrestrial (*Haemadipsa sylvestris*). Protein quantification revealed higher concentrations of crude antigens (3.60–4.14 g/dL) compared to E/S antigens (3.39–3.44 g/dl). Electrophoretic analysis showed 9 peptide bands in crude antigens (12.5-96 kDa) and five bands in E/S antigens (14-96 kDa), with no major differences in overall banding patterns. Periodic acid–Schiff (PAS) staining revealed the presence of two glycoproteins in the crude extracts of *H. manillensis* and *H. granulosa* and three in *H. sylvestris* (12.5–26 kDa). Glycoproteins could not be detected in E/S products of any of the three species. Hyperimmune serum raised in rabbits against both crude and E/S antigen was used for Enzyme immunotransfer blot (EITB) analysis, which detected two immune-reactive peptides in the crude antigens of *H. manillensis* and *H. granulosa,* and one in the E/S product of *H. sylvestris*.

Keywords: Antigen, Excretory/ Secretory, Haematophagous leech, Protein

Leeches are segmented hermaphrodites belonging to the phylum Annelida and sub-class Hirudinea. A leech may suck 5–15 mL of blood during an attachment period lasting 20–40 min, leading to injury and blood loss in its host, which can exceed the losses caused by other hematophagous parasites such as *Haemonchus contortus* (0.05 mL/worm/day), hookworm (0.09 mL/day), or *Fasciola hepatica* (0.5 mL) (Soulsby 1982). Beyond blood loss, leech bite is also associated with complications in many cases (Kose 2008, Lok *et al*. 2013). Bite of certain leeches have been reported to cause severe allergic reactions in human (Najjari *et al.* 2022). During feeding, leeches secrete a complex mixture of different biologically and pharmacologically active substances into the wound. Hirudin is a prominent active constituent of leech saliva (Singh 2010). Leeches have been widely studied for their salivary bioactive compounds, which have attracted global interest due to their therapeutic and pharmacological potential (Sawyer 1986). Leech saliva contains a complex mixture of proteins and peptides, many of which act as potent anticoagulants, platelet aggregation inhibitors, vasodilators, and anesthetics (Markwardt *et al.* 1957, Wallis 1996). Among these, hirudin, a well-characterized thrombin inhibitor, has been developed as a clinically useful anticoagulant. These bioactive molecules highlight the bioprospecting opportunities offered by leech proteins, with potential applications in cardiovascular therapy, wound healing, and even as leads for novel drug development. At the same time, characterization of immunogenic peptides is also relevant from a veterinary and medical perspective, since these proteins could serve as candidates for diagnostic markers or for prophylaxis against leech infestation.

Although the medicinal leech *Hirudo medicinalis* has been extensively studied for its salivary proteins (Alaama *et al.* 2011), limited information is available on tropical leeches such as *Hirudinaria manillensis*, reported from Malaysia and other parts of Southeast Asia (Fredric and Govedich 2004). In India, particularly in Assam and adjoining northeastern states, diverse aquatic and terrestrial leeches are abundant in wetlands, forest habitats, and livestock-grazing areas, yet their biochemical and immunological characteristics remain poorly explored. In this context, the present study focused on two aquatic species, *H. manillensis* and *H. granulosa*, and one terrestrial

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species, *Haemadipsa sylvestris*, which are among the common leeches prevalent in Assam (Pathak *et al.* 2020). *H. manillensis* and *H. granulosa* are of parasitological concern for livestock and occasionally humans, while *H. sylvestris* is often encountered in forested areas, posing nuisance and health risks to both animals and humans. Documenting the protein, glycoprotein, and immunoreactive peptide profiles of these species will provide not only the baseline information on leech biodiversity of the region but will also contribute to the broader field of leech biochemistry and host–parasite interaction research.

MATERIALS AND METHODS

Collection of leech samples: Two aquatic leech species (*Hirudinaria manillensis* and *H. granulosa*) and one terrestrial species (*Haemadipsa sylvestris*) were used in this study. The aquatic species were collected from the Deepor Beel wetlands, Guwahati, Assam (26.07°N, 91.39°E), while the terrestrial species was collected from the College of Veterinary Science, Khanapara campus, Guwahati, Kamrup district, Assam (26.14°N, 91.80°E). Collections were carried out in the early morning hours (06:00–08:00 h). Approximately 50 leeches of each species were collected. The aquatic leeches were maintained in large transparent plastic jars filled to half their volume with water with water change on alternate days, whereas the terrestrial leeches were kept in containers provided with soil from collection site at the bottom and moist cotton pads to prevent desiccation. All containers were covered with perforated lids to allow aeration. The collected specimens were subsequently transported to the ICAR–National Research Centre on Yak, Dirang, Arunachal Pradesh, for further processing.

Preparation of crude extract: For crude protein,1.5 g of leech tissue from each leech were cut from the posterior sucker and were washed five times in ice-cold phosphate-buffered saline (PBS, pH 7.2) to remove external contaminants. Tissue was cut into smaller pieces and homogenized in PBS at a ratio of 1 mL buffer per 100 mg tissue. To reduce the protease activity, anti-protease (Protease inhibitor cocktail tablet, Roche Diagnostic GmbH, Germany) was added. Tissue was homogenized using a sterile pestle and mortar and sonicated using a VCX-500 ultrasonic processor (Model VCX500) for 30 s, repeated 3–5 times at 1 min intervals, keeping samples on ice between pulses. Sonicates were aliquoted into sterile microcentrifuge tubes and centrifuged at 4°C for 15 min at 4,500×g (Thermo Scientific Multifuge X1R). The clear supernatant was collected and stored at −20 °C for future use. Three replicates of crude extract for each species were prepared.

Preparation of excretory/secretory (E/S) *protein:* Live leeches (six per replicate) were placed individually into sterile glass beakers kept on ice. Leeches were allowed to express E/S products in response to cold stress for 2 h with gentle intermittent agitation at 30 min interval. Released slimy products were collected and mixed with equal volume of ice cold PBS (pH 7.2), aliquoted and stored at -20^{0}C till further use. Three replicates of E/S antigen for each species were prepared.

Protein analysis: Total protein content of both crude and E/S antigen was estimated by Biuret method using commercially available kit (Siemens Healthcare Diagnostic Ltd., India). Protein profile of crude and E/S antigen of the respective leeches was worked out. For running the SDS-PAGE, 1X protein sample was prepared by mixing it with buffer at 1:1 ratio and a total of 20 µL volume of sample was loaded in each well. During the whole experiment denatured peptides were separated through 12.5% separating gel. For visualization of protein profile, the gels were stained by Coomassie blue stain. To visualize the glycoprotein band periodic acid Schiff (PAS) staining was done following the standard methodology described by Harlow and Lane (1988).To detect the presence of glycoprotein antigen in the crude and E/S products of leeches, PAS staining was done.

Raising of hyperimmune sera: Prior approval was obtained from the Institutional Animal Ethics Committee (Approval No. 770/ac/CPCSEA/FVSc/AAU/IAEC/13-14/207) for the use of New Zealand White rabbits in this experiment. A total of 36 healthy male rabbits (average body weight ~2.5 kg) were used for raising hyperimmune sera. For each of the three leech species (*H. manillensis*, *H. sylvestris*, and *H. granulosa*), five rabbits were immunized with crude antigens and five rabbits with E/S antigens (10 rabbits per species; 30 rabbits in total). An additional six rabbits were maintained as controls and were immunized with adjuvant-only preparations. Antigen preparations were obtained by mixing 1 mL of crude or E/S extract with 450 µL of 1 M sodium bicarbonate, followed by the drop wise addition of 0.2 M alum solution under continuous stirring until a uniform suspension was formed. The alum-precipitated antigen was allowed to stabilize at room temperature before use. Each rabbit was immunized subcutaneously with 500 µL of the prepared suspension, followed by three booster doses at weekly intervals. Seven days after the final booster dose, blood samples were collected from the marginal ear vein. Serum was separated by centrifugation and stored at –20°C until further use.

Enzyme immunotransfer blot (EITB): To evaluate the immunopeptides, enzyme immunotransfer blotting (EITB/ Immunoblotting) was performed. Proteins were separated by discontinuous SDS-PAGE as described by Laemmli (1970) and subsequently transferred onto nitrocellulose membranes (NCM; Schleicher and Schuell, Germany; pore size 0.45 µm) using a semi-dry blotting apparatus at a constant current of 50 mA for 45 min. After transfer, unbound sites on the NCM were washed with phosphate-buffered saline containing 0.05% Tween-20 (PBST). Blocking was then performed with 5% skimmed milk powder in PBST for 1 h at room temperature. The membranes were incubated with the respective rabbit hyperimmune serum (1:100 dilution) for 1 h at room temperature, followed by incubation with anti-rabbit horseradish peroxidase (HRPO)

Table 1. Protein profile, Glycoprotein profile and immune-reactive peptides of crude antigen and E/S antigen of *H. manillensis, H. granulose* and *H. sylvestris* with their relative molecular weight

Name of the species (Type of Antigen)	Protein profile with relative molecular weight (kDa)	Glycoprotein profile with relative molecular weight (kDa)	Size of immunereactive peptide
H. manillensis (Crude)	12.5, 14, 20, 22.5, 28.5, 47, 66, 68.5, 96	12.5, 20	96, 66
H. granulosa (Crude)	12.5, 14, 20, 22.5, 28.5, 47, 66, 68.5, 96	12.5, 20	66
H. sylvestris (Crude)	12.5, 14, 20, 22.5, 24.5, 26.36, 47, 66, 96	12.5, 14, 26	14, 66
H. manillensis (E/S)	14, 20, 47, 66, 96	0	96
H. granulosa (E/S)	14, 20, 47, 66, 96	0	66
H. sylvestris (E/S)	14, 26, 47, 66, 96	0	66, 47

conjugate (1:1000 dilution) for 1 h. Antigen–antibody reactions were visualized using 3,3′-diaminobenzidine (DAB) with H_2O_2, and the color development was stopped by rinsing with distilled water.

RESULTS AND DISCUSSION

The protein profiles of crude and E/S antigens from *H. manillensis*, *H. granulosa*, and *H. sylvestris* are summarized in Table 1. The total protein concentration of crude antigen was highest in *H. granulosa* (4.14 g/dL), followed by *H. sylvestris* (4.05 g/dL) and *H. manillensis* (3.6 g/dL). For E/S antigens, the values were relatively similar across species, ranging between 3.39–3.44 g/dL. Previous studies in medicinal and blood-feeding leeches documented a broader molecular weight spectrum of E/S proteins, ranging from 11–48 kDa (Baskova *et al.* 2004), 1.92–250 kDa *H. manillensis* (Abdualkader *et al.* 2013), and 17–60 kDa in other haematophagous species (Lemke *et al.* 2013). In those studies, protein band numbers often exceeded 60 (Baskova *et al.* 2004; Lemke *et al.* 2013), largely due to the use of highly sensitive approaches such as two-dimensional (2D) electrophoresis and silver staining (Baskova *et al.* 2008). In the present study, SDS-PAGE of crude antigen revealed 9–10 distinct bands for all three species, with molecular weights ranging from 12.5–96 kDa (Fig. 1). The E/S antigens yielded fewer, but 5 clear bands spanning 14–96 kDa. Although Coomassie-stained SDS-PAGE suggested discrete banding patterns, the background protein smear was also apparent, indicating the presence of numerous finer protein fractions that could not be resolved as discrete bands under the current experimental conditions. Thus, while only 9–10 major peptides were clearly documented, the gel lanes had additional faint proteinaceous components. The present study relied on discontinuous SDS-PAGE with Coomassie blue staining, a method less sensitive to low-abundance proteins. This explains why only the more prominent bands were resolved, even though finer bands were present on the gels. It is, therefore, important to emphasize that the present work does not reflect the total proteome of these leeches, but rather provides a preliminary profile of major proteins. The limitation lies not in the absence of proteins, but in the resolution and detection capability of the method employed. Advanced proteomic approaches, including peptide mass fingerprinting and LC–MS/MS, could in future identify thousands of proteins present within the bands observed here.

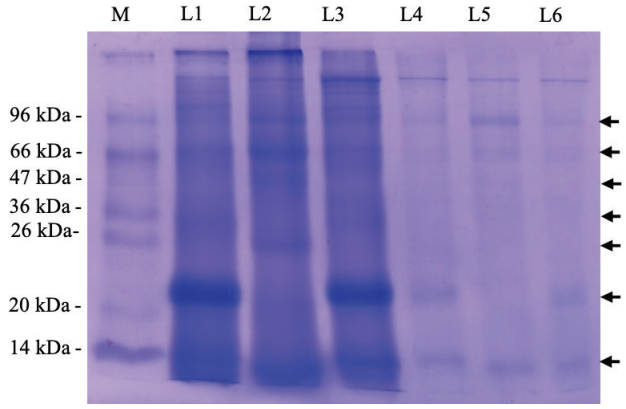

Fig.1 Protein profile of crude and E/S antigens of three leech species separated by SDS-PAGE and stained with Coomassie Brilliant Blue.Lane M: Mid-range protein marker; Lane 1: Crude extract of *H. manillensis*; Lane 2: Crude extract of *H. sylvestris*; Lane 3: Crude extract of *H. granulosa*; Lane 4: E/S extract of *H. manillensis*; Lane 5: E/S extract of *H. sylvestris*; Lane 6: E/S extract of *H. granulosa*.

PAS staining confirmed the presence of glycoproteins in the crude antigen of all species. Two glycoproteins were detected in *H. manillensis* and *H. granulosa*, whereas *H. sylvestris* crude antigen showed three glycoproteins (12.5–26 kDa; Fig. 2). In contrast, no glycoproteins could be detected in the E/S products of any species. Immunoblotting with rabbit hyperimmune sera revealed 1–2 immunoreactive peptides per preparation. In crude antigens, *H. manillensis* and *H. sylvestris* showed two reactive peptides each, while *H. granulosa* showed one (Figs. 3). For E/S antigens, one immunoreactive peptide was detected in aquatic species (*H. manillensis, H. granulosa*), and two in the terrestrial species (*H. sylvestris*). A common peptide of approximately 66 kDa was observed across preparations, except in the E/S antigen of *H. manillensis.*

Despite these limitations, this work represents first attempt to document protein, glycoprotein, and immunoreactive peptide profiles in *H. sylvestris* and *H. granulosa*, and provides partial data for *H. manillensis*. The demonstration of only immunoreactive 1–2 peptides

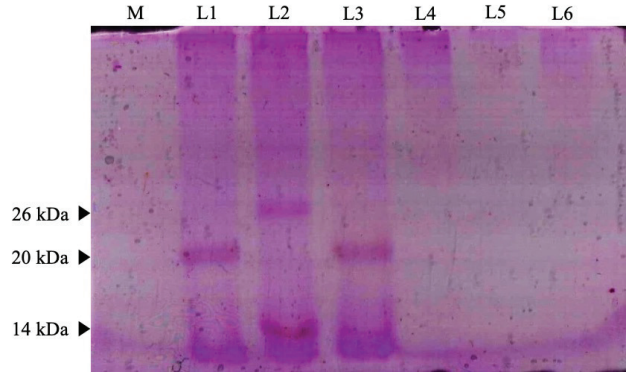

Fig. 2 PAS staining showing the presence of glycoproteins in crude and E/S antigens. Lane M: Mid-range protein marker; Lane 1: Crude extract of *H. manillensis*; Lane 2: Crude extract of *H. sylvestris*; Lane 3: Crude extract of *H. granulosa*; Lane 4: E/S extract of *H. manillensis*; Lane 5: E/S extract of *H. sylvestris*; Lane 6: E/S extract of *H. granulosa*.

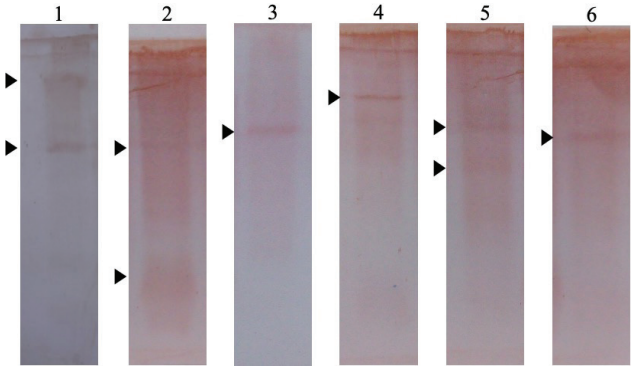

Fig. 3 Immunoblot showing immune reactive peptides in crude and E/S antigens of three leech species. 1: Crude extract of *H. manillensis* (96 and 66 kDa); 2: Crude extract of *H. sylvestris* (66 and 14 kDa); 3: Crude extract of *H. granulosa* (66 kDa); 4: E/S extract of *H. manillensis* (96 kDa); 5: E/S extract of *H. sylvestris* (66 and 47 kDa); 6: E/S extract of *H. granulosa* (66 kDa).

suggest selective recognition by the host immune system, with the 66 kDa peptide being common across preparations. This selectivity highlights potential candidates for further characterization. Future studies employing amino acid sequencing or mass spectrometry could precisely identify these immunogenic proteins and evaluate their biological roles.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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