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Heterologous expression and immunological characterization of the arginine kinase from *Haemonchus contortus*

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Abstract
In small ruminants, *Haemonchus contortus* is a major blood-feeding parasite, resulting in symptoms from minor anemia to mortality. It has caused substantial economic losses to the global sheep industry, estimated between US $42 million and US $222 million annually. Current control methods rely on anthelmintic treatment resulting in growing anthelminthic resistance. The development of immunological and molecular strategies for controlling these parasites shows promise for future research, and in this area, it has already been observed that there is ongoing research focused on developing recombinant protein-based vaccines against *H. contortus.* This study aimed to clone, express, and characterize arginine kinase (AK), an excretory/secretory (ES) protein of *H. contortus*. Hc-AK, a member of the phosphagen kinase family, plays essential roles in cellular energy metabolism in both vertebrates and invertebrates and is recognized as an immune modulator influencing pro-inflammatory cytokine responses. The complete Hc-AK gene 1036 base pairs in length was amplified from *H. contortus* cDNA and inserted into the pET-32a(+) expression vector using *Escherichia coli* TOP10 cells. For protein production, the recombinant construct was introduced into *E. coli* BL21(DE3)pLysS cells and expression was induced with 1 mM IPTG at 37 °C with shaking at 180 rpm for 2, 4, 6, and 8 hours. SDS-PAGE followed by Coomassie Brilliant Blue staining displayed a prominent band of approximately 57.4 kDa, consistent with the predicted size of Hc-AK together with an 18 kDa fusion tag. Western blot analysis employing Ni-HRP conjugate and sera from *H. contortus*-infected sheep demonstrated specific recognition of the recombinant Hc-AK (rHc-AK), whereas no reactivity was observed with sera from uninfected controls. These findings suggest that the recombinant Hc-AK protein may serve as a promising vaccine candidate against *H. contortus* infection.

Keywords: Arginine kinase, barber pole worm, excretory/secretory protein, haemonchosis

1. Introduction
Haemonchus contortus is the most significant trichostrongylid parasite affecting small ruminants (sheep and goats) in tropical and subtropical regions, representing a major obstacle to ruminant health and productivity worldwide. The ongoing and escalating emergence of resistance to all chemical control methods and the presence of chemical residues in animal products have underscored the necessity for alternative economically viable control strategies. The quest for vaccines against economically significant parasitic helminths affecting both humans and ruminants has been a longstanding objective of many researchers in immunoparasitology, and achieving vaccination that promotes lasting protective immunity would be one of the most cost-effective approaches to managing an infection. Vaccination has proven effective in enhancing animal resistance to such infections. Barbervax®, the first commercially available subunit vaccine targeting *H. contortus*, is developed using natural gut-associated glycoproteins. Nonetheless, this method, while beneficial, has a number of drawbacks, including the necessity for multiple boosters (McRae *et al.*, 2015) [6] the restricted level of protection achieved in certain instances (Giangaspero *et al.*, 2011) [4] and the ethical issues surrounding the use of infected animals to source the native antigen for vaccination. The creation of subunit vaccines for helminth parasites aimed at practical use would represent a significant advancement in managing GIN infections, especially those caused by *Haemonchus* (Matthews *et al.*, 2016) [5].

Although trials with native antigen-based vaccines yielded promising outcomes, their broader use is hindered by challenges in sourcing sufficient quantities of worm material, maintaining batch consistency, and ensuring formulation stability for commercial distribution (Broomfield *et al.*, 2020) [1]. Recombinant vaccines offer practical benefits such as standardized production, easier handling, storage, and elimination of ethical concerns.

Arginine kinase (AK), a well-conserved enzyme in the phosphagen kinase (PK) family, has been widely studied due to its high sequence homology across numerous invertebrate species, including *H. contortus*, *Trypanosoma cruzi*, *Caenorhabditis elegans*, *Heterodera glycines*, *Toxocara canis*, *Ascaris lumbricoides*, and certain proteobacteria. In these organisms, AK facilitates the reversible transfer of a phosphate group from MgATP to arginine, producing phosphoarginine and MgADP. This enzyme plays a vital role in the energy metabolism of infective *H. contortus* larvae. Moreover, AK has been identified as a component of excretory/secretory products (ESPs) in nematodes like *Teladorsagia circumcincta*, *Anisakis simplex*, and *Heligmosomoides polygyrus*. Phosphagens and their kinases in invertebrates are promising vaccine targets due to their high antigenicity and localization in metabolically active tissues such as muscles, intestines, ovaries, and uterus—whereas arginine kinase is notably absent from the cuticle.

2. Materials and Methods

2.1 Collection of adult H. contortus worms, Isolation of RNA and Synthesis of cDNA: Adult *Haemonchus contortus* worms were freshly harvested from the abomasum of a sheep at a nearby slaughterhouse and preserved in RNA*later* solution at -20°C. Total RNA was extracted from the collected worms using the RNeasy Mini Kit® (Qiagen) and subsequently stored at -80°C. For cDNA synthesis, 1 µg of the extracted RNA was used with the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). The resulting cDNA was kept at -20°C for further use.

2.2 Polymerase Chain Reaction (PCR) amplification of Hc-AK gene: The Hc-AK gene was amplified using gene-specific primers: Hc-AK Exp FP (5' GCC CAT GGA GGA CGG CTA CCA GAC TCT T 3') and Hc-AK Exp RP (5' ATA AGC TTT GTG CTT CAC TCC GTC GTA CA 3'). The PCR was carried out under the following thermal cycling conditions: initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds, and extension at 72°C for 1 minute, with a final extension step at 72°C for 10 minutes. The PCR product was analysed on a 1% agarose gel to confirm the presence of a single, specific band. Subsequently, large-scale PCR amplification was performed, and the resulting product was purified from the gel.

2.3 Restriction Enzyme (RE) digestion of PCR product and expression vector: The E. coli TOP10 cells harboring the pET-32a(+) plasmid were cultured overnight in Luria-Bertani (LB) broth at 37°C. The following day, plasmid DNA was isolated using the GeneJET Plasmid Miniprep Kit (Thermo Scientific). Both the purified PCR product and the pET-32a(+) expression vector were subjected to double digestion with the FastDigest restriction enzymes *Nco*I and *Hind*III simultaneously. Specifically, 1.44 µg of PCR product (36 ng/µl) and 2.0 µg of pET-32a(+) plasmid (144 ng/µl) were digested at 37°C for 40 minutes. The digested fragments were then purified from an agarose gel.

2.4 Transformation into Chemically competent TOP10 cells and Confirmation of recombinant clones: The restriction enzyme-digested PCR product and pET-32a(+) vector were ligated at a 3:1 molar ratio and incubated at 22°C for 2 hours, followed by overnight incubation at 4°C. Chemically competent *E. coli* TOP10 cells were prepared using the Hanahan method. Subsequently, 10 µl of the ligation mixture was transformed into the competent cells following the ice-chilled calcium chloride method, and the transformants were plated on LB agar containing ampicillin. Three colonies were randomly selected and inoculated into 5 ml of LB broth supplemented with ampicillin (final concentration 100 µg/ml), then incubated overnight at 37°C with shaking at 180 rpm. Plasmid DNA was extracted from the overnight cultures and analysed by double digestion with *Nco*I and *Hind* III restriction enzymes (Takara). To confirm successful cloning, plasmid PCR was performed using Hc-AK-specific primers under previously optimized conditions. Glycerol stocks of confirmed positive clones were prepared and stored at -80°C for future use.

2.5 Expression of recombinant Hc-AK in BL21-DE3(pLysS) strain of E. coli: Approximately 2 µl of the recombinant plasmid construct (pET-32a+/Hc-AK) was transformed into chemically competent *E. coli* BL21(DE3)pLysS cells using the previously described method. The following day, six colonies were randomly selected and screened using colony PCR to confirm the presence of the insert. Positive clones were then induced with IPTG (Isopropyl-β-D-thiogalactopyranoside) at final concentrations of 1 mM and 2 mM, respectively, and incubated at 37°C. At 2-, 4-, 6-, and 8-hours post-induction, 1 ml of culture was harvested and centrifuged at 5000 × g for 5 minutes. The resulting cell pellets were mixed with Laemmli buffer, and the protein samples were resolved on a 12% SDS-PAGE gel under native conditions. The gel was stained with Coomassie Brilliant Blue R-250 and subsequently destained to visualize protein expression.

2.6. Bulk expression and purification of recombinant Hc-AK: Protein expression was scaled up in 200 ml of LB broth under optimized conditions (1 mM IPTG induction for 6 hours at 37°C). The harvested bacterial pellet was resuspended in 15 ml of lysis buffer (pH 7.9) containing 0.5 M NaCl, 20 mM Tris-HCl, 5 mM imidazole, 1 mM PMSF, and 1 mg/ml lysozyme. This was followed by three freeze-thaw cycles and sonication using an ultra-sonicator (20 bursts of 20 seconds each with 20-second intervals). The lysate was centrifuged, and the supernatant was collected. The remaining pellet was then treated with lysis buffer containing 8 M urea to extract insoluble proteins. SDS-PAGE was performed to assess the solubility of the recombinant Hc-AK protein. The recombinant protein was further purified using Ni-NTA affinity chromatography. Eluted fractions were analyzed by SDS-PAGE, pooled, and gradually dialyzed against decreasing concentrations of urea (6 M, 4 M, 2 M, 1 M, and 0.5 M) for protein refolding.

2.7 Characterization of rHc-AK protein by Western blot with Ni-HRP probe: The dialyzed protein sample was

mixed with 5X Laemmli buffer, and SDS-PAGE was carried out in duplicate. One half of the gel was stained with Coomassie Brilliant Blue R-250 to assess the size and purity of the protein, while the other half was used for Western blot analysis. Following protein transfer, the membrane was washed and blocked overnight at 4°C using 3% BSA prepared in PBST. The next day, the membrane was washed again and incubated for 5 hours at room temperature with a 1:500 dilution of Ni-HRP conjugate (HisProbe™-HRP Conjugate, Thermo Scientific) in PBS, using a rocker for gentle agitation. Development was performed in a dark room using a DAB horseradish peroxidase chromogenic detection kit, and the reaction was stopped by rinsing the membrane thoroughly with water. The membrane was then air-dried for result analysis.

2.8. Characterization of rHc-AK by Western Blot with known positive and negative sera: Western blot was repeated using known haemonchosis-positive and -negative sera, each diluted 1:5, and incubated with the membrane for 4 hours at room temperature on a rocker. After washing, the membrane was incubated with the secondary antibody—Rabbit Anti-Sheep HRP conjugate (Invitrogen, USA)—diluted 1:750, for 2 hours at room temperature with gentle rocking. Development was carried out as previously described.

3. Results

3.1 Isolation of H. contortus RNA and synthesis of cDNA: RNA was extracted from approximately 20-30 *H. contortus* worms and eluted in 50 µl of RNase-free water. The RNA concentration was measured at 750 ng/µl. The synthesized cDNA was stored at -20°C for future use.

3.2 Amplification of Hc-AK gene and isolation of pET-32a (+) expression vector: The amplified PCR product was analyzed on a 1% agarose gel, revealing a single, specific band of the expected size, 1036 bp (Fig. 1). A large-scale PCR reaction (150 µl) was then performed, followed by gel purification, yielding a product concentration of 36 ng/µl. The pET-32a(+) plasmid was also isolated, with a concentration of 144 ng/µl and a purity ratio (A260/280) of 1.84.

3.3. Transformation and Confirmation of the recombinant clones: The restriction enzyme-digested PCR product (1032 bp) and pET-32a(+) vector (5855 bp) were purified from agarose gels. The concentrations of the digested PCR product and vector were 33 ng/µl and 11.8 ng/µl, respectively (Fig. 2). Approximately 10 µl of the ligation mixture was used to transform TOP10 competent cells. The following day, about 250-300 bacterial colonies were observed. Plasmids were extracted from three selected colonies, yielding concentrations of 167 ng/µl, 152 ng/µl, and 216 ng/µl. Double restriction enzyme digestion was performed on these plasmids, and the products were analyzed by 1% agarose gel electrophoresis (Fig. 3). All three plasmids showed the expected pattern with insert release following digestion. Recombinant clones were further confirmed by plasmid PCR, which produced a single specific band of 1036 bp. Glycerol stocks of the confirmed positive clones were prepared and stored for future use.

3.4. Expression of recombinant Hc-AK protein: For protein expression in a prokaryotic system, 2 µl of plasmid DNA (pET-32a+/Hc-AK) at a concentration of 216 ng/µl was transformed into competent *E. coli* BL21(DE3)pLysS cells. The next day, six colonies were randomly selected and screened by colony PCR, all of which showed the expected 1036 bp band confirming the presence of the Hc-AK gene (Fig. 4). Induction conditions were optimized using one recombinant clone with IPTG concentrations of 1 mM and 2 mM. Cultures were harvested at 2, 4, 6, and 8 hours post-induction. The highest expression level of the recombinant Hc-AK fusion protein (~57.4 kDa) was achieved with 1 mM IPTG after 6 hours of induction (Fig. 5). Bioinformatic analysis predicts the size of the target Hc-AK protein to be approximately 34 kDa, to which an ~18 kDa fusion tag encoded by the vector is added during expression.

3.5. Purification of recombinant Hc-AK protein: Large-scale expression of the recombinant Hc-AK protein was carried out in 200 ml of LB broth. Bacterial cells were lysed using both lysis buffer without urea and lysis buffer containing 8 M urea. As shown in Fig. 6, rHc-AK was detected in the supernatant after sonication and subsequently purified through Ni-NTA affinity chromatography. SDS-PAGE analysis confirmed the presence of the purified recombinant protein, with an approximate size of 57.4 kDa, in the eluted fractions (Fig. 7). The protein-containing fractions were pooled, dialyzed, and concentrated. Using the BCA assay, the protein concentration was determined to be 450 µg/ml.

3.6. Characterization of rHc-AK protein by Western blot with Ni-HRP probe, and known positive and negative sera: The purified protein was transferred onto a nitrocellulose membrane and probed with a Ni-HRP conjugate. Following development, a band around 57.4 kDa was detected, confirming that the rHc-AK protein was successfully expressed as a recombinant His-tagged fusion protein. To evaluate the antigenicity of the rHc-AK protein, Western blotting was performed using sera from animals known to be positive and negative for *H. contortus* infection. The membrane was divided and incubated with the respective primary and secondary antibodies. After development, a band of approximately 57.4 kDa appeared in the blot incubated with positive sheep serum, whereas no band was detected with the negative serum (Fig. 8).

Figures

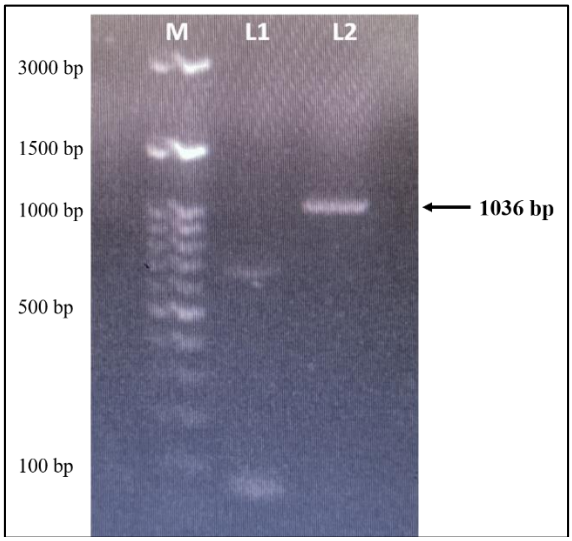

Fig 1: PCR amplification of Hc-AK gene (M - 100 bp plus DNA ladder; L1 - Positive control for PCR; L2 - PCR amplification of HcAK gene using *H. contortus* cDNA)

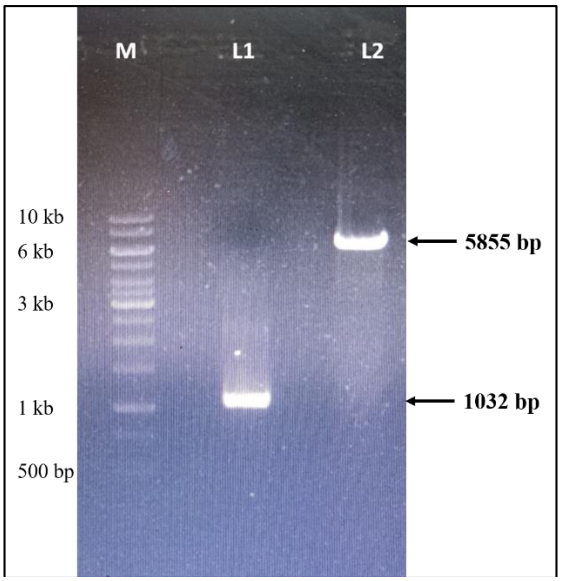

Fig 2: Restriction Enzyme digestion of purified Hc-AK PCR product and pET-32a(+) vector (M - 1kb DNA ladder; L1 - Double RE digestion of HcAK PCR product; L2 - Double RE digestion of pET-32a(+) plasmid)

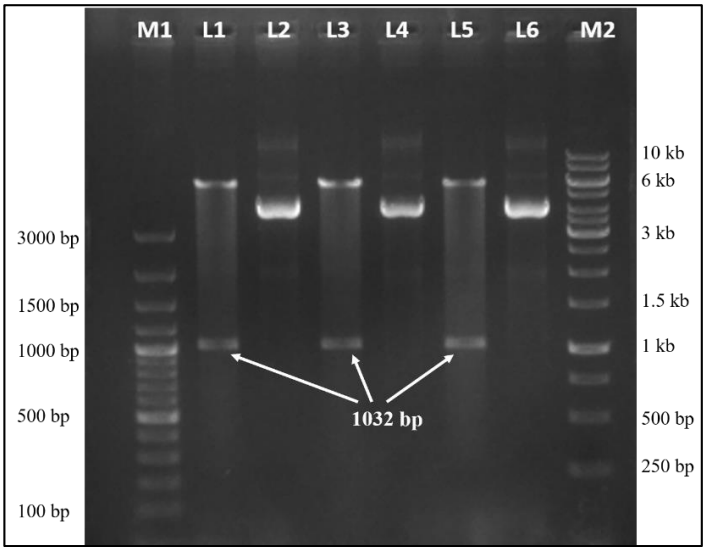

Fig 3: Confirmation of recombinant clones of Hc-AK gene by insert release (M1 - 100 bp plus DNA ladder; L1, L3, L5 - Double RE digested plasmids from transformed colonies; L2, L4, L6 - Undigested plasmids from transformed colonies; M2 - 1kb DNA ladder)

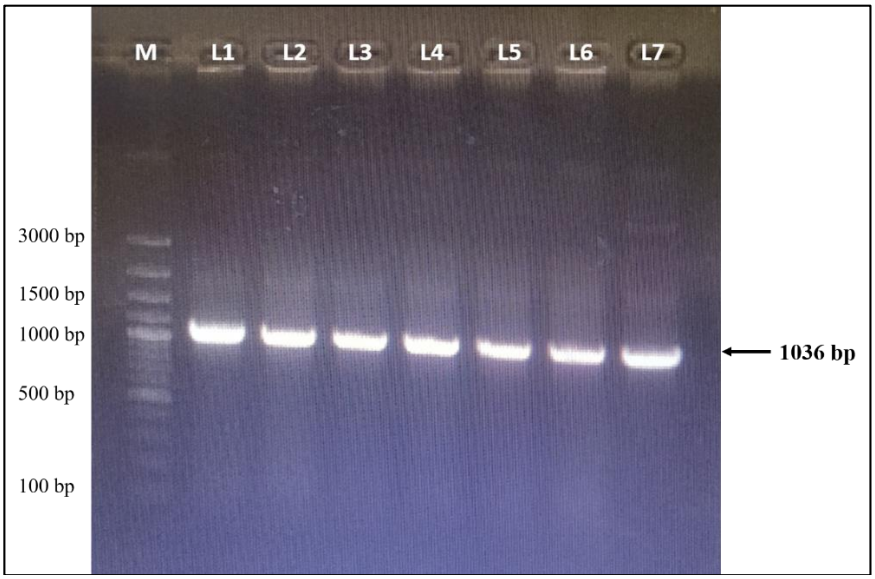

Fig 4: Confirmation of recombinant clones in BL21-DE3 (pLysS) *E. coli* by colony PCR (M - 100 bp plus DNA ladder; L1 - L6 - PCR screening of different colonies for HcAK gene; L7 - Positive control for HcAK gene)

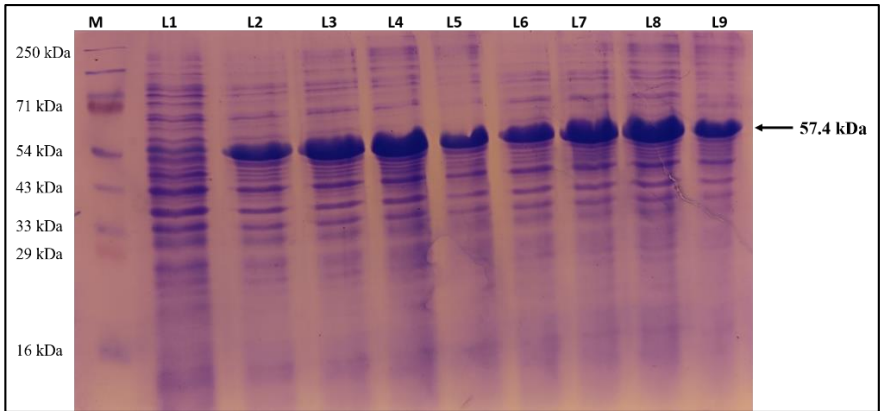

Fig 5: Expression of rHc-AK protein in recombinant clones at 2, 4, 6 and 8 hrs (M - 3 Color Prestained Protein Ladder; L1 - Uninduced recombinant clone; L2 - L5 - 1mM IPTG induction of recombinant clone for 2, 4, 6 and 8 hrs; L6 - L9 - 2mM IPTG induction of recombinant clone for 2, 4, 6 and 8 hrs)

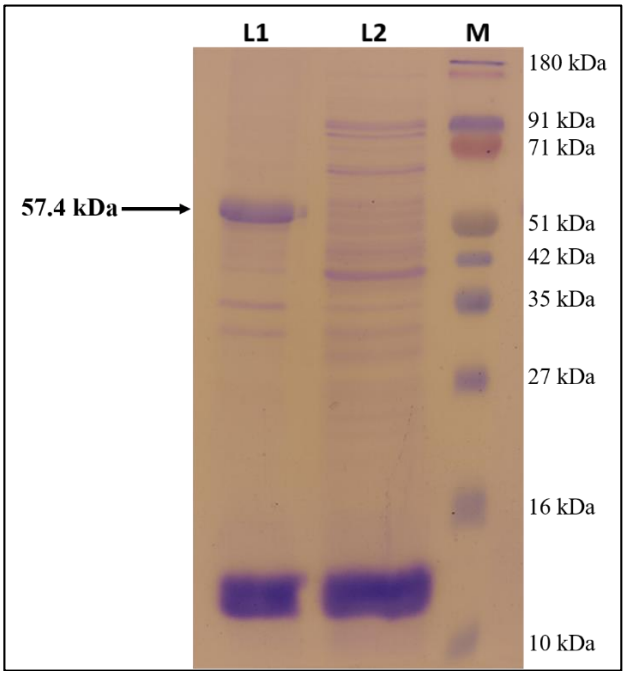

Fig 6: SDS-PAGE showing presence of rHc-AK protein in supernatant and urea treated pellet after sonication (L1 - Supernatant after sonication; L2 - Urea treated pellet after sonication; M - 4 color prestained protein ladder)

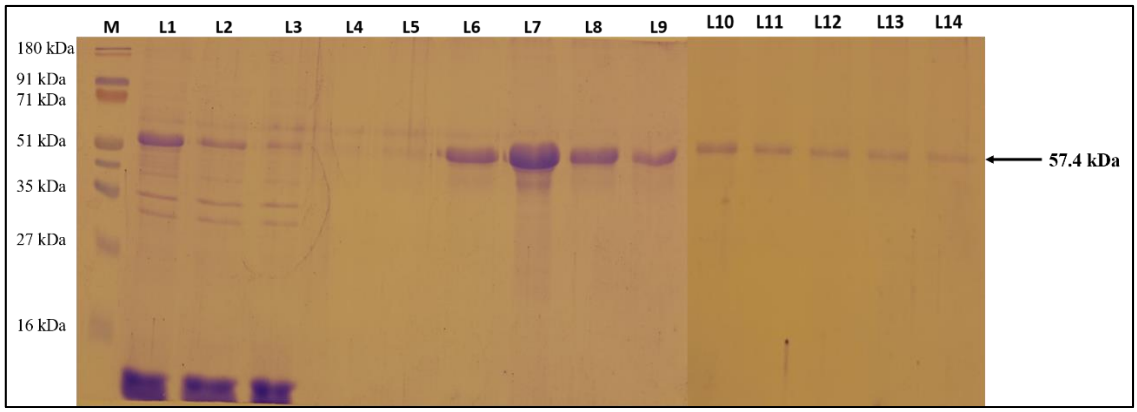

Fig 7: SDS-PAGE showing purified recombinant Hc-AK protein in different fractions (M - 4 color prestained protein ladder; L1 - Supernatant after sonication; L2 - Flow through; L3 and L4 - 1st and 2nd Wash; L5 - L9 - L14 - Elutes 1-10)

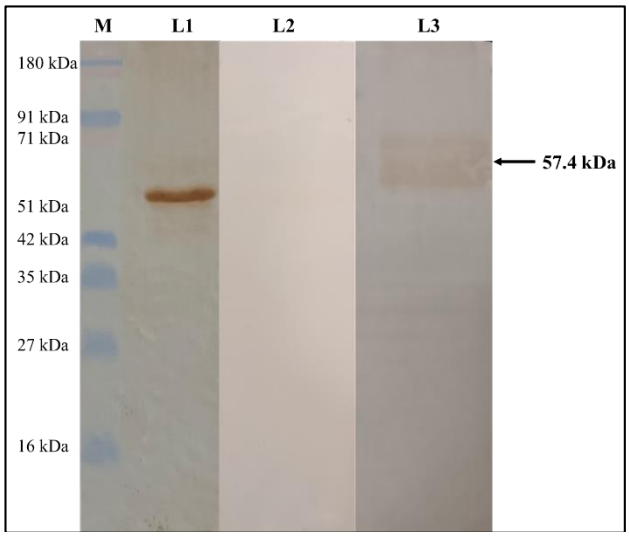

Fig 8: Western blotting of rHc-AK protein with Ni-HRP probe, *H. contortus* known negative and positive sheep sera (M - 4 color prestained protein ladder; L1 - Purified protein with Ni-HRP probe; L2 - Known *H. contortus* negative sheep sera; L3 - Known *H. contortus* positive sheep sera)

4. Discussion

Haemonchus contortus, a blood-feeding nematode inhabiting the abomasum of small ruminants, is considered one of the most destructive parasites of sheep and goats. Its impact on livestock production is profound, with annual losses estimated at US $42-222 million (Chagas *et al.*, 2022) [2]. Owing to their heightened susceptibility, sheep are often described as a "museum of parasites," reflecting the diversity of helminths they harbor (Vijayasarathi *et al.*, 2015) [9]. Clinical haemonchosis is characterized by progressive anemia, hypoproteinemia, bottle jaw, eosinophilia, and in severe infections, high mortality. The growing challenge of drug resistance has directed attention toward sustainable alternatives such as immunoprophylaxis using parasite antigens or attenuated parasites.

Energy metabolism in nematodes relies on unique biochemical pathways, providing potential molecular targets for intervention. One such system involves phosphagens, specialized guanidine compounds like creatine, taurocyamine, lombricine, and arginine, which serve as temporary energy stores. Their utilization is mediated by phosphagen kinases (PKs) that reversibly transfer phosphate between phosphagens and ATP, thereby stabilizing cellular energy balance during periods of metabolic stress. In invertebrates, including protozoa and nematodes, the predominant phosphagen system is phosphoarginine-arginine kinase (AK). Studies have shown that AK is crucial for the survival of *H. contortus* larvae by supporting high-energy demands during infection. Interestingly, these kinases are strongly antigenic in mammals and are expressed mainly in metabolically active tissues such as muscle, intestine, ovaries, and uterus, but absent from the cuticle. This selective localization enhances their appeal as vaccine candidates against parasitic nematodes (Umair *et al.*, 2013) [8].

In this study, the Hc-AK gene was cloned into the pET-32a(+) expression vector using the restriction enzymes *Nco*I and *Hind*III. The recombinant plasmid was then transformed into *E. coli* BL21(DE3)pLysS cells and expression was induced with IPTG. The recombinant Hc-AK (rHc-AK) protein was purified under native conditions using Ni-NTA agarose column chromatography. SDS-PAGE analysis showed a strong expression of the rHc-AK fusion protein with an approximate molecular weight of 57.4 kDa. Western blotting with a Ni-HRP probe confirmed that the target protein was expressed as a His-tagged fusion protein. Additionally, when probed with sera from sheep known to be positive and negative for *H. contortus* infection, the rHc-AK protein reacted specifically with positive serum, indicating its immunoreactivity. These findings suggest that the recombinant protein retains its antigenic properties after expression in a prokaryotic system.

Natural antigens of *H. contortus* include adult excretory/secretory proteins such as 15/24 kDa antigens, Hc-sL3, p26/23, arginine kinases and Hc23. Hc-AK, a significant excretory and secretory (ES) protein, is localized on both the inner and outer membranes as well as the gut region of adult worms, playing a role in the host's immune response (Ehsan *et al.*, 2017) [3]. The arginine kinase gene from *H. contortus* and *Teladorsagia circumcincta* has been cloned and expressed (Umair *et al.*, 2013) [8], with their amino acid sequences showing 99% similarity to each other and 94% similarity to the AK protein from *Caenorhabditis elegans*. Biochemical assays confirmed that the Hc-AK protein possesses enzymatic activity.

This study confirms that the arginine kinase gene exhibits immunoreactive properties, indicating its potential as a suitable immunogen. Given the widespread presence of *H. contortus* in livestock across many countries, comprehensive research to identify novel polypeptides with immunoprotective capabilities is essential for advancing vaccine development. Such studies are particularly relevant in India, where this parasite significantly impacts small

ruminant farming. This investigation is unique in successfully achieving the molecular cloning and characterization of the arginine kinase gene from an Indian isolate of *H. contortus*. Therefore, it is strongly anticipated that the recombinant Hc-AK protein could serve as a promising vaccine candidate to protect animals against *Haemonchus* infection, although further animal trials and challenge experiments are required to validate its efficacy.

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