First characterization of somatic proteins of trematodes of the family Paramphistomidae by SDS-PAGE isolated from cattle from the Cajamarca region, Peru

Charito Jennyfer Fernandez-Mendoza
Instituto Nacional de Innovación Agraria
Antony Tayca-Saldaña
Instituto Nacional de Innovación Agraria
Medali Cueva-Rodríguez
mcuevar@unc.edu.pe

Instituto Nacional de Innovación Agraria
Fernando Aliaga-Tambo
Instituto Nacional de Innovación Agraria
Cristian Hobán-Vergara
National University of Cajamarca
Claudia Rodríguez-Ulloa
National University of Cajamarca
Wuesley Alvarez-García
Instituto Nacional de Innovación Agraria
Carlos Quilcate-Pairazamán
Instituto Nacional de Innovación Agraria
Marco Cabrera-González
Instituto Nacional de Innovación Agraria

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Research

First characterization of somatic proteins of trematodes of the family Paramphistomidae by SDS-PAGE isolated from cattle from the Cajamarca region, Peru

Charito Jennyfer Fernandez-Mendoza[1] · Antony Tayca-Saldaña[1] · Medali Cueva-Rodríguez[1,*] · Fernando Aliaga-Tambo[1] · Cristian Hobán-Vergara[2] · Claudia Rodríguez-Ulloa[3] · Wuesley Alvarez-García[4] · Carlos Quilcate-Pairazamán[5] · Marco Cabrera-González[1]

[1]Laboratorio de Biotecnología en Sanidad Animal, Programa Nacional de Bovinos, Estación Experimental Baños del Inca, Dirección de Desarrollo Tecnológico Agrario, Instituto Nacional de Innovación Agraria (INIA), Baños del Inca, Cajamarca, Perú

[2]Laboratorio de Inmunología, Facultad de Ciencias Veterinarias, Universidad Nacional de Cajamarca, Avenida Atahualpa 1050, Cajamarca, Perú

[3]Escuela de Biología y Biotecnología, Facultad de Ciencias de la Salud, Universidad Nacional de Cajamarca, Avenida Atahualpa 1050, Cajamarca, Perú

[4]Estación Experimental de los Baño del Inca. Dirección de Desarrollo Tecnológico Agrario, Instituto Nacional de Innovación Agrario, Jr. Wiracocha S/N, Los Baños del Inca, Cajamarca, Perú

[5]Dirección de Desarrollo Tecnológico Agrario, Instituto Nacional de Innovación Agrario, La molina, Lima, Perú

***Correspondence**: mcuevar@unc.edu.pe

Abstract

Paramphistomosis is a gastrointestinal parasitic disease of worldwide distribution, with higher prevalence in tropical and subtropical regions. Its biological cycle has a chronic phase caused by adult trematodes that adhere to the rumen mucosa and a highly pathogenic phase caused by immature trematodes, which induce acute parasitic gastroenteritis. Studies related to the parasite are very scarce in the region of Cajamarca, Peru, regarding the characterisation of antigenic proteins, considering that this parasitosis is endemic and has emerging characteristics. The study aimed to characterise somatic

proteins of adult forms of Paramphistomidae in cattle by electrophoresis (SDS-PAGE). For this purpose, 630 adult parasites were collected from the rumen and reticulum of cattle from the processing centre of Cajamarca, Peru. We were able to characterise 21 protein bands from the somatic extract. The molecular weights of these bands ranged from 15 kDa to 119 kDa. This study may help carry out alternative control programmes such as developing vaccines, choice of appropriate drugs due to the therapeutic failures expressed by this parasite.

Keywords Cattle · Paramphistomosis · Somatic extract · SDS-PAGE · Protein (kDa)

Background

Paramphistomosis is an emerging gastrointestinal parasitic disease caused by trematodes of the family Paramphistomidae Fischoeder, 1901 [1, 2]. These parasites require freshwater pulmonate molluscs of the families Planorbidae, Bulinidae and Lymnaeidae as intermediate hosts [3] and exhibit a broad spectrum of definitive hosts from domestic ruminants such as cattle, goats, and sheep to wild ruminants [4]. They have even been documented in camelids [5] and in humans by *Gastrodiscoides hominis* Lewis and McConnall, 1876 [6–8]. Adult parasites reside in the rumen and reticulum of ruminants; however, in other species, they may be located in the large intestine or parenteral sites of ruminants, pigs and horses [9].

This disease has a global distribution, with higher prevalence in tropical and subtropical regions [10], although it also occurs in temperate zones [11]. The most affected areas include Africa, Eastern Europe, Asia, Australia and Russia [12–15]. In particular, high prevalences of 77 % have been reported in Ireland [16], 75.2 % in Sarawak-Malaysia [17], 59 % in Wales [18], 56.25 % in Pakistan [19] and 53.8 % in Northern Ireland [20]. Also, in some regions of the UK, the prevalence of paramphistomosis is higher than that of fasciolosis [2]. It has also been reported in several Latin American countries, such as Mexico [21], Brazil [22], Colombia [23], Chile [24], Peru [25], among others.

The highly pathogenic phase of the disease is caused by migrating immature trematodes, which induce acute parasitic gastroenteritis and can cause mortality [26–28]. The chronic phase, caused by adult stags attached to the rumen mucosa, reticulum or omasum [29] is less pathogenic but can cause necrosis of rumen papillae, mechanical damage to the rumen wall, ruminal atony, decreased production, etc. [19, 30, 31]. It is also essential to economic losses, affecting cattle's dairy and meat productivity [29, 32].

Diagnosis of this parasitosis remains challenging; it relies on ineffective coproparasitological examinations in situations of low parasite load or during the prepatent period of infection. Early detection in the subclinical phase is, therefore, essential for timely treatment [33]. There is currently no effective vaccine against paramphistomosis, which exacerbates the problem.

The Cajamarca region, located in the northern highlands of Peru, stands out as the country's leading dairy basin, contributing 17% of national fresh milk production [29, 34, 35]. However, the region faces the challenge of being an endemic area for this little-studied disease, with a reported prevalence of paramphistomosis at 34.2%, exceeding that of *Fasciola hepatica* Linnaeus, 1758, which is recorded at 15.3% in the district of Cajamarca [36]. The spread of this parasite is influenced by several factors, including temperature, humidity, rainfall and abundant vegetation [29, 37]. In addition, treatments are ineffective due to inappropriate administration of fasciolicides by farmers because of the similarity of symptomatology between paramphistomosis and fascioliasis [38].

Proteomics plays a crucial role in analysing proteins expressed by parasites. Information derived from proteomic approaches to parasite-host interaction, such as identifying antigenic proteins, is crucial, as these proteins can act as biomarkers in diagnosis, drug targets, and vaccine candidates [39].

Due to their immunogenicity, somatic proteins are particularly relevant in helminths [40]. For example, fatty acid binding proteins (FABPs) in *Fasciola gigantica* Cobbold, 1855, have been shown to induce damage to the parasite tegument through FABPs-based vaccines, which in turn triggers an effective immune response in the host [41]. In the somatic extract of *Gigantocotyle explanatum* Creplin, 1847, glutathione S-transferase (GST) is found in high concentrations and is an effective diagnostic antigen [42].

Several somatic proteins have been identified in paramphistomids, such as *Calicophoron daubneyi* Dinnik, 1962. Among them, fatty acid binding proteins (FABPs) are prominent in the somatic proteome, along with dehydrogenases/reductases, globins and glutathione S-transferase (GST) [43]. However, knowledge of the somatic protein profile of parasites of the family Paramphistomidae remains limited, particularly in *Calicophoron microbothrioides* Price and McIntosh, 1944, which is the species responsible for paramphistomosis in Cajamarca [29].

In this context, the present study aimed to characterise the somatic proteins of adult forms of parasites of the Paramphistomidae family in cattle by SDS-PAGE electrophoresis. This will provide a baseline

to optimise the diagnostic and control capacity of the disease and contribute to the generation of vaccines.

Materials and Methods

Ethical Statement

The research was not submitted to an ethics committee for animal experimentation because the human-animal interaction was only limited to the extraction of samples of adult parasites from the rumen and reticulum of already benefited animals after inspection by well-trained veterinarians from the Faculty of Veterinary Sciences of the National University of Cajamarca, Peru. The samples were sent to the Biotechnology in Animal Health laboratory for analysis.

Location of the study

The study was conducted at the Cajamarca cattle processing centre located in the Iscoconga village centre (7° 12' 04.6" S, 78° 26' 57.4" W), Llacanora district, Cajamarca region, Peru (Fig. 1). A total of 630 adult parasites were collected from the rumen and reticulum of cattle.

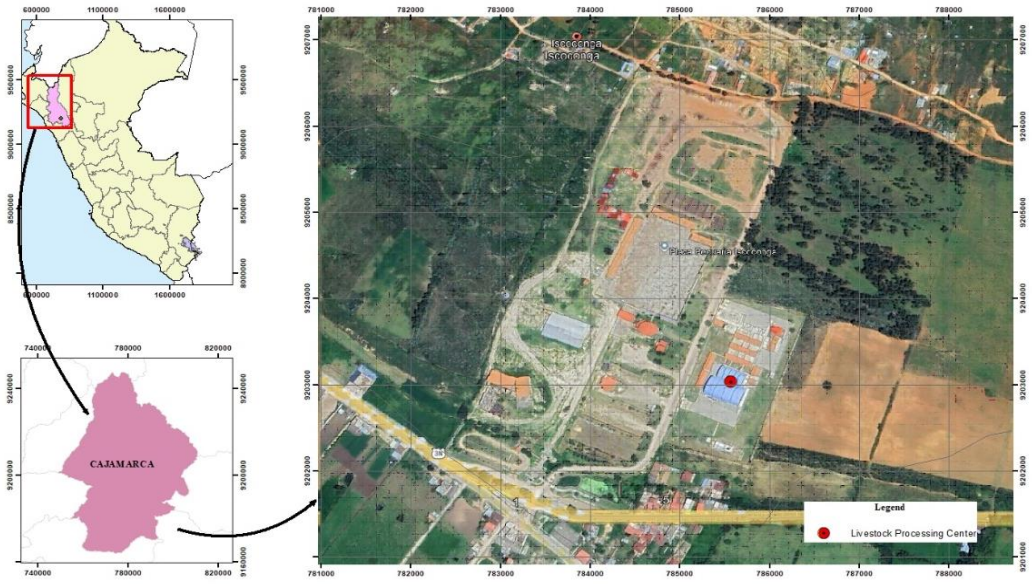

Fig. 1 Geographical location of the Cajamarca livestock processing centre. Iscoconga village centre, Cajamarca, Peru. Using ArcGIS (free version 10.8, Harvard, USA)

101 **Collection of parasites**

102 According to the methodology described by Abdolahi Khabisi and Sarkari [44], parasites were washed
103 five times with phosphate buffered saline (PBS, pH 7.2) (Thermo Scientific™, USA) and transferred to
104 50 mL tubes (Falcon®, USA) containing PBS. Subsequently, they were transported at 37 °C to the
105 Laboratory of Biotechnology in Animal Health of the Baños del Inca Agrarian Experimental Station
106 of the National Institute of Agrarian Innovation (INIA).

107 **Somatic protein extraction**

108 Somatic protein extraction was performed according to the protocol determined by Cabrera et al. [45].
109 Ten, 20, 30, and 50 parasites were distributed in individual 2 mL microtubes (Eppendorf®, Germany)
110 and preserved in an ice bucket. The microtubes were immersed in liquid nitrogen at -195 °C for
111 approximately 20 seconds, repeating this process thrice.

112 Subsequently, the microtubes were fragmented in a mortar, and the contents were crushed to a paste-
113 like consistency. The paste was collected in new 2 mL microtubes (Eppendorf®, Germany), 1.5 mL
114 PBS was added, and homogenised in a vortexer (VWR®, Vortexer Mini 230V, USA). Centrifuged at
115 14000g, 4°C, for 10 minutes (Eppendorf®, 5430 R, Germany), the supernatant was discarded, and the
116 pellet was allowed to dry at room temperature.

117 The pellet was then suspended in BugBuster Protein Extraction Reagent® (Novagen®, USA) according
118 to the manufacturer's specifications and incubated on a slow-shaking platform (Labnet International
119 Inc., USA) for 20 minutes at room temperature. One mM phenylmethylsulfonyl fluoride (Sigma®,
120 USA) was added as a protease inhibitor and incubated for 20 minutes at room temperature.

121 Insoluble cell debris was removed by centrifugation at 16000g, 4°C, for 20 minutes. The supernatant
122 was placed into a new 2 mL microtube (Eppendorf®, Germany). The protein was stored at -20 °C for
123 further analysis.

124 **Protein quantification**

125 The protein concentration of the somatic extracts was quantified using Bradford's modified method
126 Bradford [46] using a spectrophotometer (Thermo Scientific, NanoDrop™ 2000, USA). Protein Assay

Dye Reagent Concentrate (Bio-Rad, USA) was used according to the manufacturer's instructions, and bovine albumin was used as the assay standard (Spinreact®, Spain).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein extracts were analysed by SDS-PAGE as described by Cabrera et al. [45] and Alonso Villela et al. [47] with slight modifications. A 12% resolving gel and a 5% concentration gel were prepared. Proteins were denatured at 95 °C for 5 min in a water bath (Techne, Tempette TE-10D, UK), then prepared in a 1:1 ratio with sample buffer.

Subsequently, 10 µL of the broad spectrum protein marker Spectra™ Multicolor (Thermo Scientific, USA) and 29 µL of each sample were loaded into the corresponding well. Additionally, 29 µL of bovine albumin at a concentration of 250 µg/mL, with a molecular weight of ~ 66.5 kDa, was used as a control (Spinreact®, Spain). The electrophoretic run was carried out using the vertical gel electrophoresis system (Labnet International Inc., ENDURO™ VE10, USA) at a constant voltage of 50 V for 30 minutes for the concentration gel and at 120 V for 2 hours and 50 minutes for the resolution gel.

Silver staining

Gel staining was performed with the commercial Silver Stain Plus™ kit (Bio-Rad, USA), following the manufacturer's specifications.

Molecular weight determination

The somatic protein bands obtained on the gel were analysed to establish their molecular weight (MW) according to the methodology described by Matsumoto et al. [48]. Then, the relative mobility value (R_f) of the standard marker bands and each problem band was calculated in relation to the migration distance of the dye front:

$$R_f = protein\ migration\ distance\ (cm) \div gel\ front\ migration\ distance\ (cm)$$

Subsequently, the molecular weights (MW) of the standard protein marker were plotted on a base ten logarithmic scale as a function of relative mobility ($\log_{10}$ MW versus R_f), and the regression equation

was generated as follows: $y = mx + b$. Finally, the R_f-value of the protein of interest was interpolated in the obtained equation to determine its approximate MW.

Statistical analysis

Statistical analysis was performed using Graph Pad Prism 9.3.1 software (Prism Software, Irvine, CA, USA). The Kolmogorov-Smirnoff test demonstrated the data's normality. Analysis of variance tests (ANOVA) followed by a t-test were used to compare groups. Statistical differences were considered significant when the *p-value* was less than 0.05.

Results

Morphological identification of the 630 parasites collected aseptically and preserved in PBS (pH 7.2, 37 °C) was mainly located in the rumen of slaughtered cattle, which were pink or bright red in colour, conical in shape with a concave ventral face and a convex dorsal face, and were approximately 4 to 15 millimetres (mm) long by 2 to 5 millimetres (mm) wide; they also had an oral sucker and a sucker on the back surrounded by a protuberance [29].

The somatic crude extract was obtained using liquid nitrogen (-195 °C), having achieved four working concentrations with a different number of parasites, which allowed the identify the appropriate concentration for better expression and separation of protein bands by SDS-PAGE technique; these samples were analysed through the colorimetric technique for protein quantification (595 nm), so we had in concentration 1: 500 µg/mL (n = 10), concentration 2: 726.5 µg/mL (n = 20), concentration 3: 1979.5 µg/mL (n = 30) and concentration 4: 2000 µg/mL (n = 50).

Using the SDS-PAGE method with silver staining, it was observed that the number of protein bands of the paramphistomids somatic crude extract was variable according to the concentration of the protein analysed. Thus, concentration 2 showed the highest number of protein bands (21 bands) with molecular weights (MW) within a variable range of 15 kDa to 119 kDa; about the other concentrations analysed, in concentration 1, 10 bands were separated (59 kDa to 119 kDa), concentration 3, 18 bands were separated (24 kDa to 119 kDa). In concentration 4, 9 bands were separated (63 kDa to 119 kDa) (Table 1).

179 **Table 1** Protein molecular masses of paramphistomids somatic extract, according to protein
180 concentrations and number of parasites

Band number	Molecular weights (kDa)				
	Lane 1	**Lane 2**	**Lane 3**	**Lane 4**	**Lane 5**
	Ten parasites	**20 parasites**	**30 parasites**	**50 parasites**	**Control (bovine albumin 250 µg/mL)**
1	119	119	119	119	
2	113	113	113	113	
3	105	105	105	105	
4	100	100	100	100	
5	96	96	96	96	
6	91	91	91	91	66.5
7	77	77	77	77	
8	70	70	70	70	
9	63	63	63	63	
10	59	59	59		
11		56	56		
12		50	50		
13		46	46		
14		40	40		
15		38	38		
16		33	33		
17		26	26		
18		24	24		
19		23			
20		21			
21		15			

181

When analysing the protein bands on the polyacrylamide gel, a total of 09 standard bands were observed that appeared at the four concentrations used, with a variable range between 63 kDa and 119 kDa (63, 70, 77, 91, 96, 100, 105, 113 and 119 kDa) (Fig. 2).

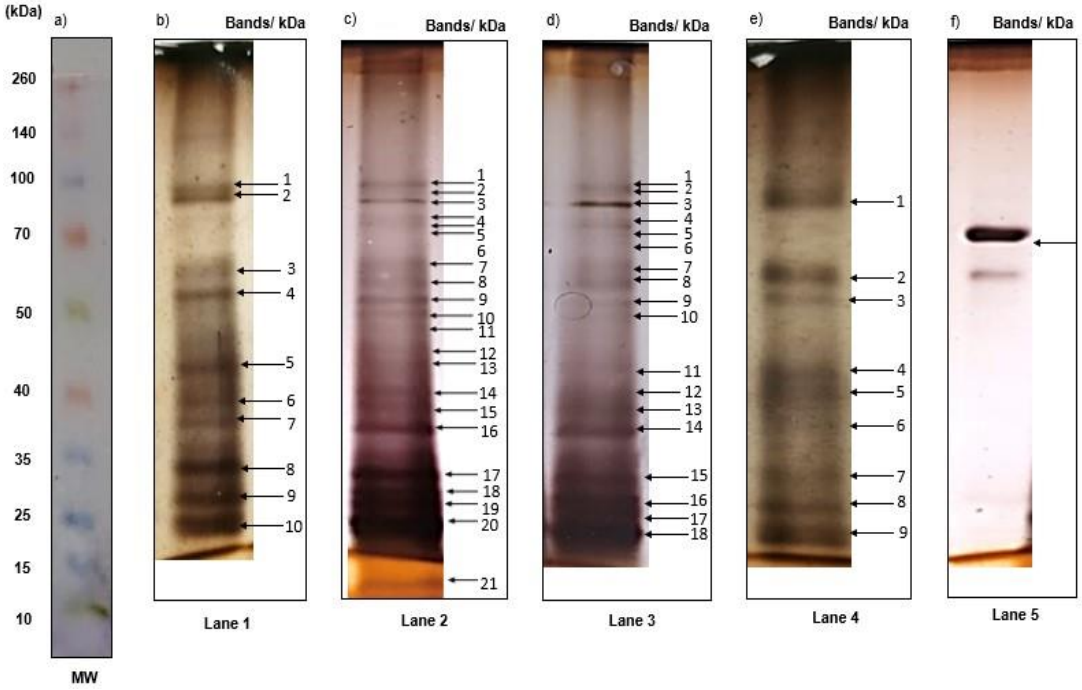

Fig. 2 Silver-stained somatic protein profile of different numbers of adult paramphistomids analysed by SDS-PAGE and their corresponding protein concentration. MW presents the molecular weight standards; lane 1 (500 µg/mL, *n* = 10); lane 2 (726.5 µg/mL, *n* = 20); lane 3 (1979.5 µg/mL, *n* = 30); lane 4 (2000 µg/mL, *n* = 50) and lane 5 (bovine albumin 250 µg/mL). Full-length gels are presented in Supplementary Figures: 2 (a); 2 (b, e); 2 (c, f) and 2 (d)

Regarding the intensity of the protein bands in the polyacrylamide gel of the different concentrations of paramphistomid somatic crude extract used, 07 protein bands were observed with molecular weights ranging from 21 kDa to 105 kDa (105, 63, 33, 26, 24, 23 and 21 kDa). In addition, 02 protein bands were shown at the four concentrations used with molecular weights of 63 kDa and 105 kDa respectively; while 03 bands were expressed at concentration 2 (726.5 µg/mL) and 3 (1979.5 µg/mL), with a molecular weight of 33 kDa, 26 kDa and 24 kDa, thus only the bands with low molecular weight of 23 kDa and 21 kDa resulted from concentration 2 (726.5 µg/mL) (Fig. 3).

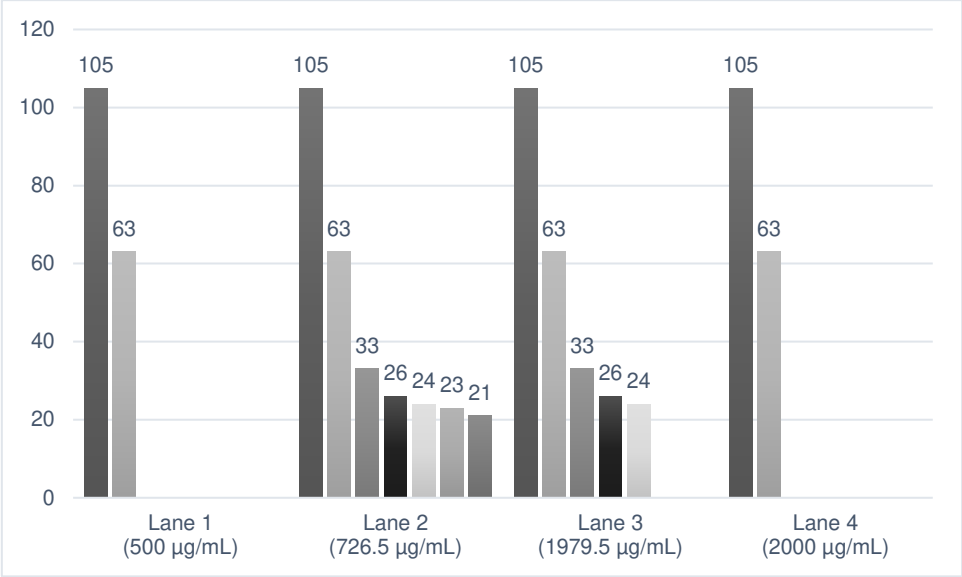

Fig. 3 Intensity of the protein bands of paramphistomids somatic crude extract, stained with silver, according to the different protein concentrations and number of parasites analysed. Lane 1 (63, 105 kDa); lane 2 (21, 23, 24, 26, 33, 63, 105 kDa); lane 3 (24, 26, 33, 63, 105 kDa); lane 4 (63, 105 kDa)

A significant difference was also found in the concentration of the somatic crude extract protein (µg/mL) and the number of separated bands (kDa) of paramphistomids ($p<0.05$), as shown in Fig. 4.

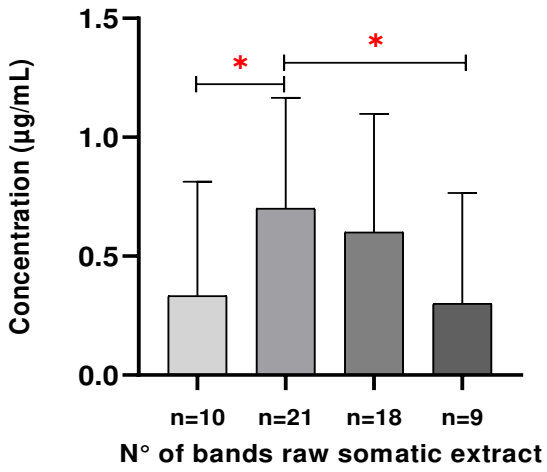

Fig. 4 Statistical analysis of paramphistomids somatic crude extract protein band. The asterisk (*) indicates a significant difference ($p<0.05$)

Discussion

The disease caused by paramphistomids is most prevalent in domestic ruminants, causing significant economic losses globally [49–51]. It is mainly distributed in tropical and subtropical areas of the world [52]. This parasite is also known as rumen stave; the adult forms are located in the rumen of the host, while the immature forms are located in the upper part of the small intestine, causing high morbidity and mortality problems of more than 90% in domestic ruminants [2, 53, 54]. In addition, it causes lesions in the intestine, leading to decreased appetite and anorexia [3, 55].

In Peru, *Paramphistomum cervi* was first described in 1975 in cattle in Iquitos [56]. Subsequently, it was reported in the district of Yurimaguas, Alto Amazonas province, Loreto region, where it presented a high prevalence favoured by environmental conditions and the lack of prevention and control strategies in cattle [57].

In Peru, according to the last National Agricultural Census - CENAGRO, the national cattle population is 5 156 000 million, of which 78% is located in the highlands, 11% in the coast and 10% in the jungle, being Cajamarca the central region of fresh milk production with 17% of the national production (2 358 000 L/year) [34], however, one of the critical activities is that Cajamarca region presents sanitary problems to diseases caused by trematodes, favoured by the presence of an appropriate ecological niche in terms of temperature and humidity, one of the critical activities of the Cajamarca region is that it presents sanitary problems to diseases caused by trematodes, favoured by the presence of an appropriate ecological niche in terms of temperature and humidity, as well as being endemic also to diseases little attended as paramphistomosis which has an importance in livestock because it represents characteristics of emerging and little studied in the region and at Latin American level [58].

Despite its condition as a parasitic disease with particular characteristics, this parasite causes high morbimortality levels in cattle; it is necessary to mention that in this region, few investigations have been carried out in this regard, such as parasite incidence and correlation of parasite load with the presence of adult forms [59]. It is, therefore, essential to increase knowledge by determining the protein profile of somatic crude extract by electrophoresis in polyacrylamide gels of adult forms of paramphistomids for analysis and characterisation. Thus, the protein profile of paramphistomids by SDS-PAGE obtained in the present study was similar to related trematodes such as *Fasciola hepatica,*

where 12 protein bands with variable molecular weight (26, 37, 40, 49, 55, 66, 70, 85, 95, 145, 185, 263 kDa) were obtained from adult forms [60].

Similarly, Abdolahi Khabisi and Sarkari [44] conducted a study with *Fasciola hepatica* and *Fasciola gigantica* where they reported something similar to what was found in our work where they determined that 30 protein bands were revealed ranging from 18 kDa to 180 kDa and with band intensities between 22.5 kDa, 27 kDa, 31 kDa and 62 kDa molecular weight, which varies with what was observed in this study where protein bands were obtained with a molecular weight from 15 kDa to 119 kDa (15, 21, 23, 24, 26, 33, 38, 40, 46, 50, 56, 59, 63, 70, 77, 91, 96, 100, 105, 113 and 119 kDa), possibly due to the difference in trematode species.

In the present study, protein characterisation of somatic crude extract of adult forms of paramphistomids was performed by polyacrylamide gel electrophoresis and many more protein bands (n = 21) of different molecular weight ranging from 15 kDa to 119 kDa (15, 21, 23, 24, 26, 33, 33, 38, 40, 46, 50, 56, 59, 63, 70, 77, 91, 96, 100, 105, 113 and 119 kDa) could be observed at the concentration of 726.5 µg/mL of protein relative to the other concentrations tested (500 µg/mL, 1979.5 µg/mL and 2000 µg/mL), possibly due to saturation of the pore diameter of the polyacrylamide gel and protein migration.

These results found are different in terms of the number of bands obtained by Salib et al. [61], who were able to find 14 protein bands of adult *Paramphistomum sp.* from cattle by electrophoresis, which have a variable molecular weight between 11.5 kDa to 174 kDa (11.5, 13.5, 19, 25, 29, 46, 52, 63, 66, 72, 87, 105, 120 and 174 kDa) and from Arora et al. [62] where they found a somatic antigen protein profile of *Paramphistomum epiclitum* composed of 14 protein bands within a range of 14.1 kDa to 95.5 kDa (95.5, 81.8, 70.8, 54.6, 51.6, 43.4, 39.8, 37.6, 35.5, 33.1, 23.7, 21.8, 16.8 and 14.1 kDa); possibly to differences in species used in the respective research works.

When analysing the results obtained, they agree with the findings of Anuracpreeda et al. [63] where they report a profile of adult *Paramphistomum cervi* composed of 26 protein bands separated by SDS-PAGE electrophoresis with a variable molecular weight between 11.5 kDa and 200 kDa, in addition, it was similar to that reported by Meshgi et al. [64] who obtained the presence of 21 protein bands between 25 kDa and 150 kDa at an optimal concentration of 8 µg/mL, which is lower than the amount

used in the present study reported at 726.5 µg/mL, a difference possibly due to the method used, amount of parasites processed, local strains and different environmental conditions.

Conclusion

It is concluded that 21 protein bands from the somatic extract of adult forms of parasites of the family Paramphistomidae in cattle were characterised using the SDS-PAGE electrophoresis technique. These bands had molecular weights ranging from 15 kDa to 119 kDa (15, 21, 23, 24, 26, 33, 38, 40, 46, 50, 56, 59, 63, 70, 77, 91, 96, 100, 105, 113 and 119 kDa), obtained from the protein concentration of 726.5 µg/mL, which was the most optimal compared to the other concentrations analysed. The results obtained from this study contribute to the control of paramphistomosis through improved diagnosis and vaccine development.

Abbreviations

SDS-PAGE: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis; FABPs: fatty acid binding proteins; GST: glutathione S-transferase; PBS: phosphate buffered saline; MW: molecular weight.

Author contribution

The study was conceptualized by MCG. Methodology CJFM and ATS. Validation FAT. Formal analysis, MCG and CRU. Internal research WAG. Resources CQP. Original draft writing MCG and CHV. Drafting, revising and editing, MCR and MCG. Project administration CQP. All authors have read and accepted the published version of the manuscript.

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Data availability

No datasets were generated and analysed during the current study.

Declarations

Ethics approval

The research was not submitted to an ethics committee for animal experimentation because the human-animal interaction was only limited to the extraction of samples of adult parasites from the rumen and reticulum of already benefited animals after inspection by well-trained veterinarians from the Faculty of Veterinary Sciences of the National University of Cajamarca, Peru. The samples were sent to the Biotechnology in Animal Health laboratory for analysis.

Consent for publication

All authors have read and approved the final manuscript and accepted its publication.

Competing interests

The authors declare no competing interests.

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