Ross University School of Veterinary Medicine
One Health Center for Zoonoses and Tropical Veterinary Medicine

Universidad de Granada, Instituto de Biotecnología
Departamento de Parasitología

Dual Doctoral Thesis
PhD by Research - RUSVM
Programa de Doctorado en Biología Fundamental y de Sistemas – UGR

# *Trichuris trichiura*: Assessment of potential alternative diagnostic methods and efficacy of treatments in the African Green Monkey animal model (*Chlorocebus sabaeus*).

Presented by:
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Opting for the PhD degree

Supervisors
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The results from the doctoral thesis have been presented in

Cruz, K., Kelly, P., Vandenplas, M., Trelis, M., Marcilla, A. (2019, August 31 - September 1). Exploring how helminth extracellular vesicles (EVs) influence African green monkeys in St. Kitts, West Indies. Parasitic Helminths: New perspectives in Biology and Infection. Helminth EV Workshop. Hydra, Greece.

Cruz, K., Marcilla, A., Kelly, P., Vandenplas, M., Osuna A., Trelis, M. (2019, July 10 – 13). Salivary Diagnostics for the human whipworm (*Trichuris trichiura*), exploring the usefulness of the humoral response. ACACPMT Congress 2019. St. Kitts, West Indies.

Cruz, K., Trelis, M., Vandenplas, M., Marcilla, A., Osuna A., Verocai, GG., Kelly, P. (2019, April 7-10). 100 years later: Molecular characterization of *Primasubulura* sp. on St. Kitts, West Indies. Molecular Helminthology Conference, An Integrated Approach. San Antonio, Texas, USA.

Cruz, K., Marchi, S., Vandenplas, M., Beierschmitt, A., Trelis, M., Marcilla, A., Osuna, A. (2017, Octubre 27 - 31). Potencialidad del modelo animal: Mono Verde Africano (*Chlorocebus Sabaeus*) - *Trichuris trichiura* para el desarrollo de nuevos métodos inmunológicos de diagnóstico y seguimiento de la infección. Sociedad Española de Medicina Tropical y Salud Internacional Bilbao, España.

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Cruz, K., Corey, T. M., Vandenplas, M., Trelis, M., Osuna, A., & Kelly, P. J. (2021). Case report: Control of intestinal nematodes in captive *Chlorocebus sabaeus*. *The Onderstepoort Journal of Veterinary Research*, *88*(1).

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*To mom, Luci and Luis, for their resilience and unconditional love.*

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Luis and Luci, you are the foundation and light of our little family, and this is a merit to us. For leaning on each other as we grew and for the amazing life we have created together.

## Extended Summary – English

There are limited data on the efficacy of antiparasitic treatments and husbandry methods that can be used to control nematode infections in captive populations of African Green Monkeys (AGMs); *Chlorocebus sabaeus.* In routine Fecal Egg Count (FEC) testing, and post-mortem helminth examinations we determined the nematodes present in AGMs captured from the large (up to 50,000) feral population on the island of St. Kitts, West Indies. The most common nematode species identified in the AGMs FEC were *Capillaria* sp., followed by *Trichuris trichiura*, and strongylid species specifically (hookworm and *Trichostrongylus* spp.), but also *Strongyloides fuelleborni*.

After obtaining the data on parasitization by nematodes, we evaluated the anthelmintic efficacy of two different treatment regimens and the husbandry methods routinely employed at the animal facility. The first treatment regimen evaluated was praziquantel (5 mg/kg, given intramuscular) and albendazole (15 mg/kg, given orally) repeated after 2 weeks (PRAL), and the second one was ivermectin (300 µg/kg, given subcutaneously) and albendazole (10 mg/kg, given orally) daily for 3 days (IVAL). Both regimens were repeated at 3 – 5 month intervals. The PRAL treatment results had limited effect with only 2/12 (17%) AGMs negative at 2 months post-treatment, 1/7 (14%) at 4-5 months post-treatment and none (0%) negative at 10 to 24 months post initial treatment. The IVAL treatment regimen was more effective with 7/11 (64%) of the recipients negative at 3 months after the first treatment. The four that remained infected were cleared of *T. trichiura* after the second round of treatment but three appeared to have been reinfected with *Capillaria*.

Husbandry methods to control reinfection consisted of a daily cage washing with water and biweekly cage sanitation using a combination of steam (>82 ˚C), a quaternary-based disinfectant and an accelerated hydrogen peroxide foam. The facility's water is sanitized, filtered, and treated with ultraviolet light; therefore, thorough washing of fruits and vegetables, maintenance of pest control practices and controlled monitoring of social enclosure times could further limit potential sources of reinfection that can impact optimal health and behavior of study animals. It is important to note that regular FECs will not detect all nematode infections and full advantage should be

taken of necropsied animals for parasite detection and surveillance. Our results support and confirm that for *T. trichiura* there is no correlation between fecal egg counts and worm counts.

Worldwide, most cases of trichuriasis remain undiagnosed, chronic infections remain undetected and an overall lack of understanding of potential zoonotic exposures prevails. Therefore, early diagnosis of trichuriasis and diagnostic methods that do not rely on inconsistent clinical signs or fecal analysis are crucial to detect infections following accidental ingestion and during prolonged prepatent period of the parasite. The difficulty of access to the parasitic material (feces and adult nematodes) and biological samples from the hosts, further threatens the development of alternative diagnostic methods. With our work, we show that the St. Kitts green monkey being naturally infected with *T. trichiura*, serves as a predictive disease modeling test system that facilitates preclinical evaluation having similar immunological responses as humans.

Embryonated eggs are the infectious developmental stage of *T. trichiura* and are the primary stimulus for the immune system of the host. The intestinal-dwelling *T. trichiura* affects an estimated 465 million people worldwide with an estimated global burden of disease of 640,000 DALYs (Disability Adjusted Life Years). In Latin America and the Caribbean, trichuriasis is the most prevalent soil transmitted helminthiasis in the region (12.3%). The adverse health consequences impair childhood school performance and reduce school attendance, resulting in lower future wage-earning capacity. The accumulation of the long-term effects translates into poverty promoting sequelae and a cycle of impoverishment.

Each infective *T. trichiura* egg carries the antigens needed to face the immune system with a wide variety of proteins present in the shell, larvae's surface, and the accompanying fluid that contains their excretions/secretions. We used a proteomic approach with tandem mass spectrometry to investigate the proteome of soluble non-embryonated Egg Extracts (EE) of *T. trichiura* obtained from naturally infected AMGs. A total of 231 proteins were identified, 168 of them with known molecular functions. The proteome revealed common proteins families which are known to play roles in energy and metabolism; the cytoskeleton, muscle and motility; proteolysis; signaling; the stress response and detoxification; transcription and translation; and lipid binding and transport. In addition to the study of the *T. trichiura* non-embryonated egg proteome, the antigenic profile of the *T. trichiura* EE and soluble Female Extract (FE) proteins against serum and saliva antibodies

from *C. sabaeus* naturally infected with trichuriasis was investigated. We used an immunoproteomic approach by Western blot and tandem mass spectrometry from the corresponding SDS-PAGE gels. Vitellogenin N and VWD and DUF1943 domain containing protein, poly-cysteine and histidine tailed protein isoform 2, carbohydrate-Binding Module 14 domain containing protein, kunitz bovine basic pancreatic trypsin inhibitor domain containing protein and heat shock protein 70, glyceraldehyde-3-phosphate dehydrogenase, actin, and enolase, were among the potential immunoactive proteins.

To identify possible cross-reactions of *T. trichiura* and other helminthic antigens, Western blots were performed on soluble protein extracts from other helminths, nematodes (*Primasubulura* sp., *Anisakis simplex, Aspiculuris tetraptera*) and trematodes (*Fasciola hepatica, Dicrocoelium dendri*ticum and *Gnathosoma hispidium*) with serum from *T. trichiura* naturally infected AGMs.

A total of four synthetic peptides were designed based on the amino acid sequences deducted from the candidate proteins present in the EE and, FE and recognized by saliva and serum antibodies from *C. sabaeus* naturally infected with *T. trichiura*. The final candidate proteins from which the peptides were designed were: Vitellogenin N and VWD and DUF1943 domain containing protein, CBM 14 domain containing protein, Kunitz BPTI domain containing protein, Poly-cysteine and histidine tailed protein isoform 2. The diagnostic feasibility of the selected peptides derived from the four final protein candidates VgNVD (YRSLAIDDVLTETAP), KBDCP (QERCVATLPASVICRLP), CBM14 (SAPTTVTTQLSPVDCVTL), and PCHTP-2 (TECVKPPAHDCPAFG) was evaluated with 19 serum and 19 saliva samples from *T. trichiura* naturally infected AGMs and 6 negative human sample controls.

In both, the saliva and the serum samples, the peptides that elicited the strongest recognition by antibodies were the KBDCP (QERCVATLPASVICRLP), which is an FE stage specific protein of low abundance, and the VgNVD (YRSLAIDDVLTETAP), which is an EE stage specific protein. The egg protein CBM14 (SAPTTVTTQLSPVDCVTL), and the most abundant protein in all developmental stages of the parasite PCHTP-2 (TECVKPPAHDCPAFG) presented the weakest signals (optical density, OD). Overall, the saliva samples showed strongest optical density values and lower cutoff values than the serum samples.

To our knowledge, this is the first study on the *T. trichiura* non-embryonated egg proteome as a novel source of information on potential targets for immunodiagnostics and immunomodulators from a neglected tropical disease. This initial list of *T. trichiura* non-embryonated egg proteins (proteome and antigenic profile) can be used in future research on the immunobiology and pathogenesis of human trichuriasis and the treatment of human intestinal immune-related diseases.

Existe escasez de información acerca de la eficacia de los tratamientos antiparasitarios y los métodos de vigilancia y seguimiento que deben usarse para controlar las infecciones por nematodos en poblaciones cautivas de monos verdes africanos (African Green Monkeys; AGM); *Chlorocebus sabaeus.* En los ensayos para el diagnóstico parasitario en heces (Fecal Egg Count; FEC), así como en los exámenes de intestino grueso realizados post-mortem para la identificación de presencia o ausencia de helmintos, logramos cuantificar la presencia de nematodos en AGMs capturados de una gran población salvaje (aproximadamente 50.000) en la isla de St. Kitts, Antillas Menores. Las especies de nematodos más comunes identificadas en las heces fueron *Capillaria* sp., seguida de *Trichuris trichiura*, especies de strongylidos (anquilostoma y *Trichostrongylus* spp.), y también *Strongyloides fuelleborni*.

Después de obtener los datos sobre la parasitación por nematodos, evaluamos la eficacia antihelmíntica de dos regímenes de tratamientos diferentes, así como los métodos de cuidado y mantenimiento empleados rutinariamente en la institución donde residen los animales. El primer régimen de tratamiento evaluado fue praziquantel (5 mg/kg, intramuscular) y albendazol (15 mg/kg, oral) repetido a las 2 semanas (PRAL), y el segundo método fue ivermectina (300 μg/kg, subcutáneo) y albendazol (10 mg/kg, oral) a diario por 3 días (IVAL). Ambos regímenes se repitieron a intervalos de 3 a 5 meses. Los resultados del tratamiento de PRAL tuvieron un efecto limitado con solo 2/12 (17%) AGMs con resultados negativos a los 2 meses post-tratamiento, 1/7 (14%) a los 4-5 meses post-tratamiento, y ninguno negativo de los 10 a los 24 meses desde del tratamiento inicial. El régimen de tratamiento IVAL fue más eficaz con 7/11 (64%) de los AGMs negativos a los 3 meses después del primer tratamiento. Los cuatro AGMs que permanecieron infectados lograron eliminar la infección con *T. trichiura* después de una segunda tanda de tratamiento, sin embargo, tres AGMs aparecieron reinfectados con *Capillaria* sp.

Los métodos de estabulado de los animales empleados para controlar la reinfección consistieron en el lavado diario de las jaulas con agua y la desinfección con vapor (>82˚C), y un desinfectante de base cuaternaria y espuma de peróxido de hidrógeno cada dos semanas. En la institución, el agua se desinfecta, se filtra y se trata con luz ultravioleta, junto con ello, el lavado de frutas y

vegetales, el mantenimiento de las buenas prácticas de control de plagas y el monitoreo de los tiempos en jaulas de grupos, podrían limitar aún más las posibles fuentes de reinfección que pueden afectar la salud y el comportamiento óptimo de los animales. Es importante recalcar que a pesar de realizarse exámenes de heces frecuentes no se detectarán todas las infecciones por nematodos y se debe aprovechar al máximo a los animales sometidos a necropsia para la detección y vigilancia parasitaria. Nuestros resultados apoyan y confirman que para *T. trichiura* no existe una correlación entre el número de huevos en el diagnóstico en heces, por los métodos tradicionales de conteo de huevos, y el número de parásitos en el intestino grueso.

A nivel mundial, la mayoría de casos de trichuriasis permanecen sin diagnosticar, las infecciones crónicas no son detectadas, y prevalece una falta general de comprensión en cuanto a su potencial zoonótico. Por lo tanto, el diagnóstico temprano de trichuriasis unido a métodos de diagnóstico que no se basen en signos clínicos inespecíficos o únicamente en análisis de heces son cruciales para detectar infecciones accidentales especialmente durante el prolongado periodo prepatente del parásito. La dificultad de acceso al material parasitario (heces y nematodos), y a muestras biológicas de los hospedadores, complica aún más el desarrollo de métodos de diagnóstico alternativos. Con nuestro trabajo, demostramos que el mono verde de St. Kitts, al estar naturalmente infectado con *T. trichiura*, facilita su uso como un modelo para la evaluación preclínica de la enfermedad al tener una respuesta inmunológica similar a la de los humanos.

Los huevos embrionados de *T. trichiura* son el estadio infectivo de este nematodo y representan el primer estímulo para el sistema inmunitario del hospedador. El parásito intestinal *T. trichiura* se estima que afecta a 465 millones de personas a nivel mundial, con una carga de enfermedad de 640.000 DALYs (años de vida ajustados a la discapacidad). En América Latina y el Caribe, *T. trichiura* es el geohelminto más prevalente de la región (12.3%). Los consecuencias adversas para la salud afectan al rendimiento escolar de la niñez y reducen la asistencia a la escuela, lo que resulta en una menor capacidad para obtener ingresos en el futuro adulto. La acumulación de efectos a largo plazo se traduce en secuelas promotoras de pobreza y en un ciclo de empobrecimiento.

Cada huevo embrionado de *T. trichiura* contiene los antígenos necesarios para enfrentarse al sistema inmunitario, una gran variedad de proteínas presentes en su cubierta, en la superficie de

la larva que contiene y en el líquido de excreción/secreción que acompaña a los componentes del huevo. Se utilizaron herramientas de proteómica mediante espectrometría de masas en tándem para investigar el proteoma de las proteínas solubles del extracto del huevo no embrionado (EE) de *T. trichiura* obtenido de AGM infectados naturalmente. Se identificaron un total de 231 proteínas, 168 de ellas con funciones celulares conocidas. El proteoma reveló familias de proteínas comunes, que son conocidas por su relación con la obtención de energía, en el metabolismo; el citoesqueleto, el músculo y la movilidad; la proteólisis; la señalización; la respuesta al estrés y la detoxificación; la transcripción y la traducción; y la unión de lípidos y el transporte celular. Además del estudio del proteoma del huevo de *T. trichiura*, se investigó el perfil antigénico de las proteínas solubles, tanto del huevo (EE) como de la hembra (FE) de *T. trichiura,* sobre los anticuerpos presentes en el suero y en la saliva de *C. sabaeus* naturalmente infectados con trichuriasis. Para ello, se utilizó un enfoque immunoproteómico con *Western blot* y espectrometría de masas en tándem de los geles SDS-PAGE correspondientes. La proteína vitellogenina N con dominios de VWD y DUF1943, la proteína poli-cisteína e histidina isoforma 2, la proteína con dominio de módulo de unión a carbohidratos 14, la proteína kunitz con dominio de inhibidor de la tripsina pancreática bovina,  la proteína de choque térmico 70 (HSP-70), la gliceraldehido-3-fosfato deshidrogenasa, la actina y la enolasa, son las proteínas propuestas en nuestro estudio como las potencialmente immunoactivas.

Para identificar posibles reacciones cruzadas entre antígenos de *T. trichiura* y otras especies de helmintos, se realizaron Western blots con extractos de las proteínas solubles de otros helmintos, nematodos (*Primasubulura* sp., *Anisakis simplex, Aspiculuris tetraptera*) y trematodos (*Fasciola hepatica*, *Dicrocoelium dendri*ticum and *Gnathosoma hispidium*) contra suero de AGM naturalmente infectados con *T. trichiura*.

Un total de cuatro péptidos sintéticos se diseñaron basados en las secuencias de aminoácidos deducidas de las proteínas candidatas presentes en EE y FE, y que eran potencialmente reconocidas por anticuerpos de saliva y suero de *C. sabaeus* naturalmente infectados con *T. trichiura*. Las proteínas candidatas finales de donde fueron diseñados los péptidos son: vitellogenina N con dominios de VWD y DUF1943 (VgNVD), la proteína la proteína con dominio de módulo de unión a carbohidratos 14 (CBM14), la proteína kunitz con dominio de inhibidor de la

tripsina pancreática bovina (KBDCP) y la proteína poli-cisteína e histidina isoforma 2 (PCHTP-2). La capacidad diagnostica de los péptidos derivados de las cuatro proteínas candidatas VgNVD (YRSLAIDDVLTETAP), KBDCP (QERCVATLPASVICRLP), CBM14 (SAPTTVTTQLSPVDCVTL), and PCHTP-2 (TECVKPPAHDCPAFG) fue evaluada contra 19 muestras de suero y 19 muestras de saliva de AGM naturalmente infectados con *T. trichiura,* y 6 muestras de saliva y suero control negativas de humano.

En ambos grupos de muestras, saliva y suero, los péptidos que provocaron el mayor reconocimiento por anticuerpos fueron KBDCP (QERCVATLPASVICRLP), una proteína específica de la hembra adulta (FE) y de poca abundancia y la VgNVD (YRSLAIDDVLTETAP) una proteína específica del huevo (EE) de *T. trichiura*. Otra proteína encontrada en el EE de *T. trichiura* CBM14 (SAPTTVTTQLSPVDCVTL), y la proteína más abundante en todos los estados de desarrollo del parásito, la PCHTP-2 (TECVKPPAHDCPAFG) presentaron las respuestas más débiles (densidad óptica, OD). En resumen, las muestras de saliva obtuvieron los valores de densidades ópticas mas pronunciados, y los valores de cut-off más bajos en comparación con las muestras de suero.

Hasta la fecha, este es el primer estudio del proteoma de los huevos de *T. trichiura* y el uso de estos como una fuente alternativa de información sobre posibles moléculas diana para immunodiagnóstico e immunomodulación de esta enfermedad tropical desatendida.

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## List of Abbreviations, Acronyms, and Initialisms

| Abbreviation | Meaning |
|---|---|
| 1-DE | One Dimensional Electrophoresis |
| 2-DE | Two Dimensional Electrophoresis |
| AAALAC | Association for Assessment and Accreditation of Laboratory Animal Care International |
| ACN | Acetonitrile |
| AE | Adult Extract |
| AGM | African Green Monkey |
| APV | Association of Primate Veterinarians |
| ATP | Adenosine Triphosphate |
| BE | Equilibration Buffer |
| BLAST | Basic Local Alignment Search Tool |
| BSA | Bovine Serum Albumin |
| CBM14 | Carbohydrate-Binding Module 14 domain containing protein |
| CDC | Centers for Disease Control and Prevention |
| CEGMA | Core Eukaryotic Genes Mapping Approach |
| CHAPS | Cholamidopropyl dimethylammonium-1-propanesulfonate |
| CIC | Center for Scientific Instrumentation |
| CIDA | Chronic Iron Deficiency Anemia |
| DALYs | Disability Adjusted Life Years |
| DNA | Deoxyribonucleic Acid |
| DTT | Dithiothreitol |
| EE | Egg Extract |
| EGF | Epidermal Growth Factor |
| ELISA | Enzyme Linked Immunosorbent Assay |
| EMBL-EBI | European Bioinformatics Institute |
| EPG | Eggs Per Gram |

| | |
|---|---|
| ERR | Egg Reduction Rates |
| ES | Excretion/Secretion |
| EVs | Extracellular Vesicles |
| FA | Formic Acid |
| FASTA | Fast-All |
| FE | Female Extract |
| FEC | Fecal Egg Counts |
| GAPDH | Glyceraldehyde-3-Phosphate Dehydrogenase |
| GO | Gen Ontology |
| HSP-70 | Heat Shock Protein 70 |
| IAA | Iodoacetamide |
| IACUC | Institutional Animal Care and Use Committee |
| IDA | Information Dependent Acquisition Mode |
| IEF | Isoelectric Focusing |
| IFN-γ | Interferon γ |
| IL | Interleukin |
| IRB | Institutional Review Board |
| ITS | Internal Transcribed Spacers |
| IVAL | Ivermectin and Albendazole |
| KBDCP | Kunitz Bovine Basic Pancreatic Trypsin Inhibitor (BPTI) domain containing protein |
| LAMP | Loop-Mediated Isothermal Amplification |
| LC-MS/MS | Liquid Chromatography and Tandem Mass Spectrometry |
| MDA | Mass Drug Administration |
| MS/MS | Tandem Mass Spectrometry |
| MSP | Major Sperm Proteins |
| MW | Molecular Weight |
| NCBI | National Center for Biotechnology Information |

| NE | Non-Embryonated |
|---|---|
| NHP | Nonhuman Primate |
| NTDs | Neglected Tropical Diseases |
| OD | Optical Density |
| OPD | O-Phenylenediamine dihydrochloride |
| PBS | Phosphate Buffered Saline |
| PBST | PBS - Tween |
| PCHTP-2 | Polycysteine and Histidine Tailed Protein isoform 2 |
| PCR | Polymerase Chain Reaction |
| PO | *Per orem* "by mouth" |
| PRAL | Praziquantel and Albendazole |
| RUSVM | Ross University School of Veterinary Medicine |
| SCSIE | Servei Central de Suport a la Investigació Experimental |
| SD | Standard Deviation |
| SDS-PAGE | Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis |
| SEM | Scanning Electron Microscopy |
| SKBRF | Saint Kitts Biomedical Research Foundation |
| SLPI | Secretory Leukocyte Protease Inhibitor |
| SQ | Subcutaneous |
| STH | Soil-Transmitted Helminth |
| TDS | *Trichuris* Dysentery Syndrome |
| TFA | Trifluoracetic Acid |
| $T_H1$ | T helper Lymphocyte 1 |
| $T_H2$ | T helper Lymphocyte 2 |
| $T_H9$ | T helper Lymphocyte 9 |
| VgNVD | Vitellogenin N and VWD and DUF1943 domain containing protein |
| WAP | Whey Acidic Protein |
| WB | Western Blot |

| | |
|---|---|
| WHO | World Health Organization |
| YLDs | Years Lived with Disability |

## List of Appendices

Appendix I          Karnovsky's fixative

Appendix II         Double centrifugation with Sheather's sugar flotation solution

Appendix III        Standard Operating Procedure for saliva collection

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# 1  Introduction

## 1.1  Human helminthiasis: Nematoda (roundworms)

Key concepts

- Soil-transmitted helminth (STH) infections are a major group of nematodes amongst the most common infections in humans worldwide.

- Mainly caused by the whipworm *Trichuris trichiura*, *Ascaris lumbricoides* and the hookworms; *Necator americanus* and *Ancylostoma duodenale.*

- They infect the host by accidental ingestion of eggs present in human or nonhuman primate (NHP) feces or by active infections after skin penetration of the larvae (*N. americanus* and *A. duodenale*).

- Eggs and larvae can contaminate food, water, and soil, especially in areas where sanitation is poor.

- Approximately 1.5 billion people are infected with STH worldwide (Pullan *et al.*, 2014).

- 4.98 million years lived with disability (Years Lost to Disability (YLDs)) are attributed to STH (Pullan *et al.*, 2014).

- Infected children are nutritionally, physically, and cognitively impaired. During adulthood, they will face reduced worker productivity and quality of life.

- Control is based on periodical deworming through Mass Drug Administration (MDA) campaigns, preventive health education and improved sanitation conditions.

- Diagnosis is based on the detection of eggs in feces using a variety of fecal analysis techniques.

- *Strongyloides stercoralis* is also part of the STH but requires different diagnostic methods and treatment, for this reason, is usually underrepresented, underdiagnosed, and not impacted by MDA programs as the other STH.

Human parasitic helminths are classified into three main groups as represented in **Figure 1.1** Nematodes, trematodes and cestodes.
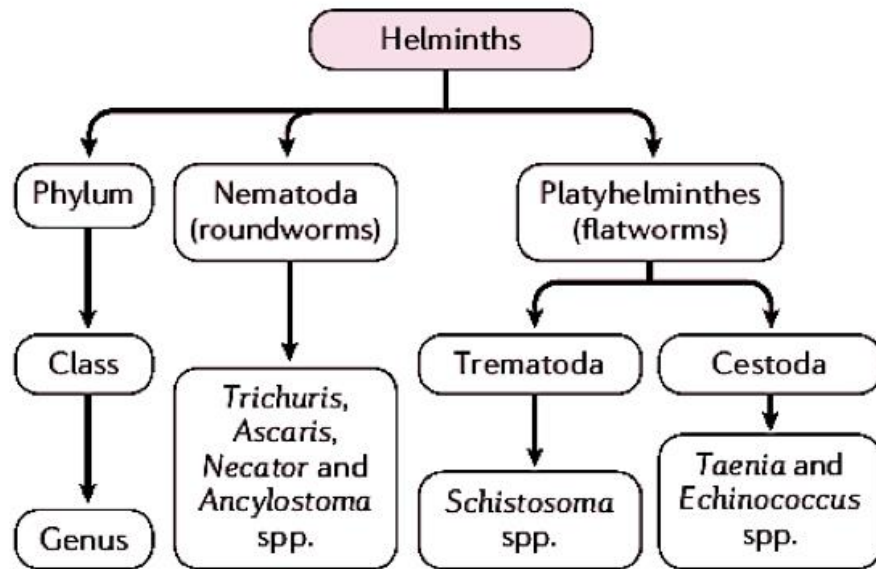
**Figure 1.1 Nematoda, Soil-Transmitted Helminths (STH) in the perspective of helminth family.**

Adapted from Else *et al*., 2020

Human helminthiasis is an umbrella term for these multicellular endoparasites, and STH are the most common nematode infections in humans.

### 1.1.1 Soil-Transmitted Helminths (STHs): Global burden and relevance in public health

Globally an estimated 2000 million people are infected with STHs (World Health Organization (WHO), 2018) and the number of global deaths associated with high-intensity infections adds up to 12,000 to 135,000 per year (Salaam-Blyther *et al*., 2011; WHO, 2012b). Infections are broadly distributed in all continents, but almost 70% of the infections occur in Asia (Pullan *et al*., 2014). *Ascaris lumbricoides* shows the widest distribution with the highest transmission rate observed in Cameroon (30.8%); hookworms (*Ancylostoma duodenale* and *Necator americanus*) highest transmission observed in the Central African Republic (30.5%), and *T. trichiura* infections reached the highest prevalence in Malaysia (49.9%) (**Figure 1.2 and Table 1.1).**
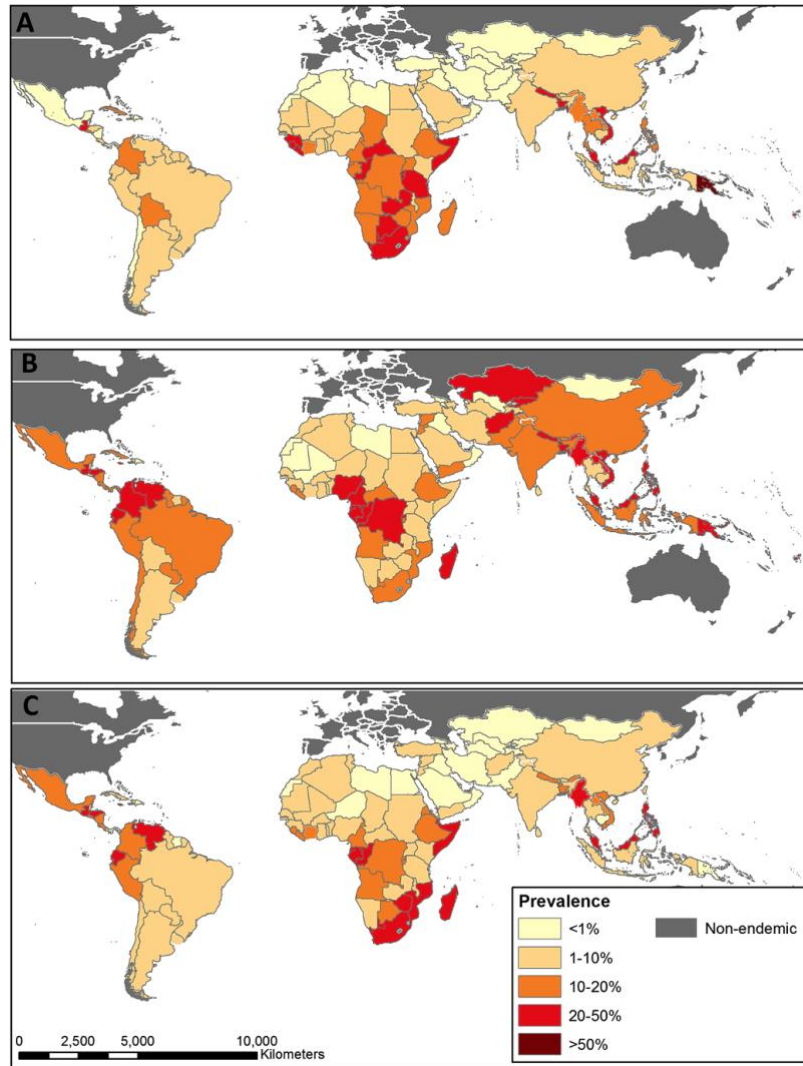
**Figure 1.2 Distribution of STHs infection prevalence in 2010 by parasitic species.**
(A) Hookworm, (B) *Ascaris lumbricoides* and (C) *Trichuris trichiura*; based on geostatistical models for sub-Saharan Africa and available empirical information for all other regions (Pullan *et al*., 2014).

Human STHs have been a part of our life's since before the era of our earliest recorded history, their eggs can be found in mummified feces of humans dating back thousands of years (Evans, 1996; Hotez *et al*., 2008; Else *et al*., 2020). They are a part of the 20 major Neglected Tropical Diseases (NTDs) identified by the World Health Organization. Although they are endemic in low-middle income developing countries of Africa, Asia, and America with warm, humid tropical or subtropical climates, developed countries are also affected in socioeconomically disadvantaged groups (WHO, 2018; Mutombo *et al*., 2019).

Table 1.1 Estimated global numbers infected with soil transmitted helminths and overall prevalence.

**Table 4 Estimates of global numbers infected with soil-transmitted helminths in 2010, by region**

| REGION | Total population (millions) | Infected Populations in millions (95% CI[1]) | | | Overall prevalence (95% CI) | | |
|---|---|---|---|---|---|---|---|
| | | Hookworm | A. lumbricoides | T. trichiura | Hookworm | A. lumbricoides | T. trichiura |
| **Asia** | **3736.7** | **281.8 (249.5-318.5)** | **589.0 (524.4-660.3)** | **282.3 (248.5-323.5)** | **7.5% (6.7-8.7%)** | **15.8% (14.5-17.7%)** | **7.6% (6.6-8.7%)** |
| Central Asia | 80.7 | 0.1 (0.01-0.2) | 6.0 (5.1-6.9) | 0.1 (0.2-.25) | 0.1% (0.0-0.3%) | 7.4% (6.4-8.5%) | 0.1% (0.0-0.3%) |
| East Asia | 1424.4 | 64.5 (44.9-87.3) | 158.4 (124.7-194.1) | 66.2 (41.9-93.5) | 4.5% (3.3-5.5%) | 11.1% (8.8-13.6%) | 4.6% (3.0-6.6%) |
| South Asia | 1621.1 | 140.2 (117.2-173.0) | 297.8 (263.8-345.4) | 100.7 (80.3-129.8) | 8.7% (5.2-6.6%) | 18.4% (16.3-21.9%) | 6.2% (5.0-8.0%) |
| Southeast Asia | 610.5 | 77.0 (69.2-84.9) | 126.7 (116.0-137.4) | 115.3 (106.8-125.3) | 12.6% (11.3-13.9%) | 20.8% (19.0-22.5%) | 18.9% (17.5-20.5%) |
| **LAC** | **586.0** | **30.3 (25.5-35.5)** | **86.0 (78.2-95.6)** | **72.2 (66.0-80.0)** | **5.2% (4.4-6.1%)** | **14.7% (13.4-16.3%)** | **12.3% (11.3-13.7%** |
| Caribbean | 39.7 | 2.1 (1.81-2.36) | 3.2 (2.8-3.7) | 2.8 (2.5-3.1) | 5.2% (4.5-5.9%) | 8.1% (7.0-9.4%) | 7.0% (6.3-7.7%) |
| Andean LA | 52.7 | 2.3 (1.73-2.91) | 10.6 (9.2-12.3) | 10.3 (9.0-12.0) | 4.3% (3.3-5.5%) | 20.1% (17.5-23.3%) | 19.6% (17.1-22.7%) |
| Central LA | 230.3 | 13.5 (12.04-15.15) | 41.8 (38.1-45.7) | 44.0 (40.4-47.6) | 5.9% (5.2-6.6%) | 18.1% (16.6-19.9%) | 19.1% (17.6-20.7%) |
| Southern LA | 57.9 | 1.4 (1.00-1.92) | 5.9 (5.1-7.0) | 2.1 (1.5-2.7) | 2.5% (1.7-3.3%) | 10.2 (8.7-12.2%) | 3.5% (2.5-4.8%) |
| Tropical LA | 205.4 | 11.0 (6.83-15.77) | 24.5 (18.0-32.5) | 13.0 (8.1-19.4) | 5.4% (3.2-7.7%) | 11.9% (8.7-15.8%) | 6.4 (3.9-9.5%) |
| **SSA** | **866.0** | **117.7 (111.1-125.9)** | **117.9 (108.7-127.1)** | **100.8 (94.1-108.0)** | **13.6% (12.9-14.6%)** | **13.6% (12.6-14.8%)** | **11.6% (10.9-12.6%** |
| Central SSA | 98.0 | 19.3 (16.5-22.2) | 21.0 (17.8-24.7) | 16.5 (13.6-20.1) | 19.7% (16.6-22.6%) | 21.4% (18.1-25.2%) | 16.9% (13.9-20.5%) |
| East SSA | 358.7 | 49.5 (45.7-54.3) | 34.4 (30.3-38.8) | 42.2 (37.9-46.8) | 13.8% (12.8-15.2%) | 9.6% (8.5-10.9%) | 11.8% (10.6-13.1%) |
| Southern SSA | 70.4 | 14.9 (12.9-17.3) | 8.6 (6.7-10.7) | 23.3 (20.7-26.0) | 21.2% (19.1-25.8%) | 12.2% (10.1-5.9%) | 33.1% (30.8-38.7%) |
| West SSA | 339.0 | 34.0 (30.0-38.9) | 53.9 (46.7-60.7) | 18.8 (15.3-23.2) | 10.0% (8.9-11.5%) | 15.9% (13.8-17.9%) | 5.5% (4.5-6.8%) |
| **North Africa and Middle East** | **477.4** | **4.6 (4.0-7.1)** | **24.3 (22.6-28.5)** | **8.7 (7.3-10.7)** | **1.0% (0.9-1.6%)** | **5.4% (531–6.4%)** | **1.9% (1.6-2.4%)** |
| **Oceania** | **9.6** | **4.6 (4.3-3.8)** | **1.9 (1.6-2.2)** | **0.6 (0.6-0.7)** | **47.9% (44.7-51.0%)** | **19.7% (16.6-23.1%)** | **6.4% (5.8-7.0%)** |
| **GLOBAL[2]** | **5,631.4** | **438.9 (406.3-480.2)** | **819.0 (771.7-891.6)** | **464.6 (429.6-508.0)** | **7.8% (7.2-8.5%)** | **14.5% (13.7-15.8%)** | **8.3% (7.6-9.0%)** |

[1]Credible interval, based on within-admin2 variation generated by Bayesian linear mixed model. LAC, Latin America and the Caribbean. SSA, sub-Saharan Africa.
[2]Global prevalence includes populations in Asia, LAC, SSA, North Africa and the Middle East and Oceania as the denominator.

Highest overall worldwide in Latin America and the Caribbean (12.3%) highlighted in red (From Pullan *et al*., 2014).

The intestinal-dwelling *T. trichiura* affects an estimated 465 million people worldwide with a global burden of disease of 640,000 DALYs (Disability Adjusted Life Years) (Pullan *et al*., 2014) and 337,000 YLDs (Years Lost to Disability) (Vos *et al*., 2017), although lower estimates have also been recently reported (James *et al*., 2018). In Latin America and the Caribbean, STHs are present in all countries with an estimated 26.3 million school-age children at risk of infection with an overall prevalence of trichuriasis which is the highest in the region (12.3%) (**Table 1.1**) (Lustigman *et al*., 2012; Pullan *et al*., 2014).

The adverse health consequences of STHs impair childhood school performance and reduce school attendance, resulting in a lower future wage-earning capacity (Stephenson *et al*. 2000; Humphries *et al*., 2021). The accumulation of the long-term effects translates into poverty promoting sequelae that explains why poor communities remain in a cycle of impoverishment (Hotez *et al*., 2008).

Children of school years represent the majority of clinically significant cases, and worm

burden tends to decline significantly in adulthood (Else *et al.*, 2020). This decline of infection burden in adulthood has been related to a combined acquired immunity and/or a lower exposure to infection due to changes in behavior with an improvement in hygiene habits (Stephenson *et al.*, 2000; Hotez *et al.*, 2008, Humphries *et al.*, 2021).

### 1.1.2 Soil-Transmitted Helminths strategies for control

During the 2001 World Health Assembly, the resolution WHA54.19 was endorsed urging endemic countries to increase their efforts for controlling STHs. The strategies focus on morbidity control through the implementation of periodic Mass Drugs Administration (MDA) campaigns integrated with school health programmes for populations at risk. Treatment campaigns target preschool and school-age children, women of reproductive age, including pregnant women in the second and third trimesters, breastfeeding women, and adults in high-risk occupations (WHO, 2018). Also, the health and hygiene education campaigns help reduce transmission and reinfection by encouraging healthy behaviors and by finding alternatives despite not always having adequate sanitation, which is a concern in resource-poor settings. In 2018, endemic countries treated over 676 million school-aged children (53% of all children at risk), following the United Nations guidelines endorsed by the WHO regarding STH global targets by 2030 (WHO, 2018):

1. Achieve and maintain the elimination of STH morbidity in pre-school and school-age children.
2. Reduce the number of tablets needed in preventive chemotherapy for STH.
3. Increase domestic financial support to preventive chemotherapy for STH.
4. Establish an efficient STH control programme in adolescents, pregnant and lactating women.
5. Establish an efficient strongyloidiasis control programme in school-age children.
6. Ensure universal access to at least basic sanitation and hygiene by 2030 in STH-endemic areas.

A large volume of research has explored social, environmental, and behavioral determinants of STH infection; however, understanding correlations between these factors can

provide some new ideas in terms of strategies for control. For example, large scale variability in the prevalence and distribution of *T. trichiura* together with other co-infections is important for defining control programs based on location-specific treatment needs (Sturrock *et al.*, 2017). While countries with the highest prevalence of multiple NTDs, including trichuriasis, may benefit from MDA programmes as a rapid impact approach, other countries, with a more scattered distribution of trichuriasis, and regional variability such as the Americas would be better candidates for targeted treatment efforts with previous diagnosis and determination of worm burdens (Lustigman *et al.*, 2012).

Additionally, recent reviews of randomized controlled trials questioned the effectiveness of current MDA programs and policies (Taylor-Robinson *et al.*, 2015, 2017; Jourdan *et al.*, 2018). These reviews concluded that the population-level effect of deworming was absent on a range of child health outcomes such as growth and hemoglobin levels (Taylor-Robinson *et al.*, 2015, 2017; Jourdan *et al.*, 2018). Others caution that elimination of STHs transmission can have an unintended but significant impact on the increased risk of autoimmune diseases, a phenomenon known as the hygiene hypothesis (Jouvin *et al.*, 2012; Wammes *et al.*, 2014).

### 1.1.3 Zoonotic potential of STHs and reservoirs

The zoonotic potential of STH infections and the variety of reservoirs has been a topic of great debate. Molecular analyses have made it possible to identify potential zoonoses with a higher degree of confidence, especially when morphological differences between eggs, larvae, or adult nematodes does not allow for species identification (Nejsum *et al.*, 2012). The overall consensus is that further sampling of species from humans, NHPs, and domesticated animals, such as dogs and pigs, from different geographic distributions, combined with molecular analysis is warranted to continue determining the extent and importance of the potential STHs zoonotic transmissions (Betson *et al.*, 2015; Mutombo *et al.*, 2019).

In the specific case of trichuriasis, most human cases are attributed to *T. trichiura,* but reports of human infection with *Trichuris vulpis* (Areekul *et al.*, 2010), the species of canids, and *Trichuris suis* (Summers *et al.*, 2005), the species of swine, were confirmed using molecular techniques. Under experimental conditions, *T. suis* cross-infection can occur, and although infections seem

to establish only temporary (Nejsum *et al*., 2012), one study found fully grown adults in a study volunteer (Williams *et al*., 2017). The complexity of *T. trichiura* phylogenetic relationships between humans and NHPs is explained in detail in the genomics section (**1.2.4 Genomics and Proteomics**).

## *1.2    Trichuris trichiura*

### 1.2.1   Classification and Taxonomy

*Trichuris trichiura* under the helminth umbrella, belongs to the phylum Nematoda and the genus *Trichuris.*

Phylum: Nematoda

Class: Enoplea

Order: Trichinellida

Superfamily Trichinelloidea

Family: Trichuridae

Genus: *Trichuris*

Species: *Trichuris trichiura* (Linnaeus, 1758).

The genus *Trichuris* belongs to the class Enoplea and the order Trichinellida together with *Trichinella spiralis.* A superfamily of nematodes, including the following roundworms that are parasitic in humans: *Trichinella spiralis*, the trichina worm (family Trichinellidae); *T. trichiura*, the human whipworm; *Capillaria hepatica*, the capillary liver worm; and *Capillaria philippinensis*.

Over 70 *Trichuris* spp. are recognized in different mammalian hosts, including the etiological agent of trichuriasis, *T. trichiura* and *T. suis* in pigs are the most closely related species (Betson *et al.*, 2015). Although the scientific name *Trichuris* given by Johann Georg Roederer in 1761 means, "hair tail", the thin portion of the parasite's body is the head (Else *et al*., 2020). While various authors have used the term *Trichocephalus* which is morphologically precise, the correct taxonomic denomination is *Trichuris* (Acha *et al*., 2003).

## 1.2.2 Life cycle and Transmission

*Trichuris trichiura* life cycle as a soil-transmitted helminth, with equal routes of transmission, mainly fecal-oral, in humans and nonhuman primates is shown in **Figure** 1.3.
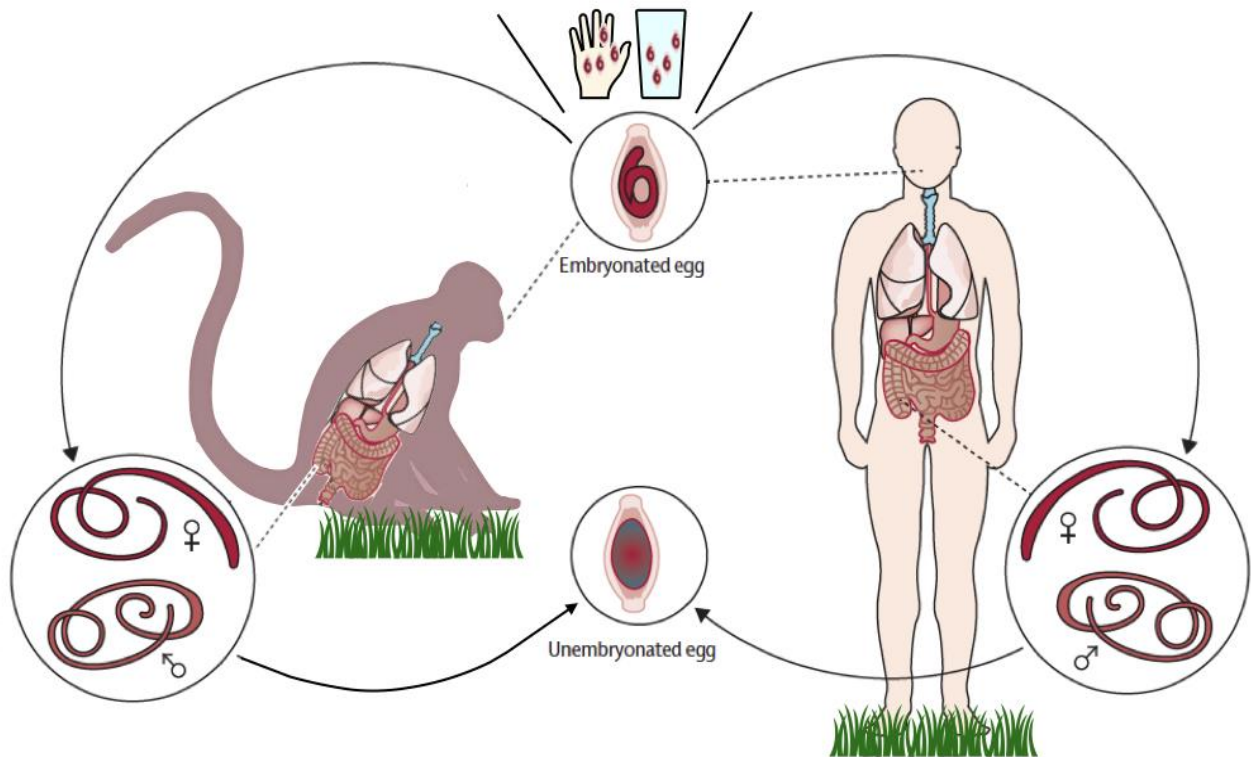
**Figure 1.3 Life cycle of *Trichuris trichiura.***
Adapted from Jourdan *et al*. (2018).

After the accidental ingestion of the embryonated eggs from contaminated hands, food, soil, or water, larvae (L1) hatch in the proximal small bowel and migrate aborally to the colon and cecum where they penetrate the epithelial cells at the crypts of Lieberkühn base, creating an intracellular niche where they undergo four molts (L2, L3, L4). However, by the L3 stage, the parasite is no longer fully intracellular, because as it grows in length, it reemerges to the intestinal lumen, but remains attached to the mucosa (Bundy and Cooper, 1989; Else *et al.*, 2020). The last larvae (L4) mature to adults in 4 to 16 weeks, survive from 1 to 8 years, and measure $3-5$ cm (Bundy and Cooper, 1989; Hansen *et al*., 2016; Else *et al*., 2020). Their thick posterior ends are free in the intestinal lumen while their anterior part remains attached to the mucosa, surrounded

by a syncytial tunnel within the epithelium cells, which biology remains unknown (Else *et al*., 2020). After copulation, the adult females oviposit thousands of eggs: 2-20,000 eggs/day (Bundy and Cooper, 1989); 18,000 eggs/female per day (Hansen *et al*., 2016), expelled in their unembryonated (not infectious) form in the feces. To embryonate, they require 2-4 weeks in the appropriate soil and/or ambient conditions that allow the development of the larvae into the infectious developmental stage: the embryonated egg containing L1 larvae (Else *et al*., 2020). *T. trichiura* eggs measure 50-60 µm in length and 20-30 µm in width, they do not develop in direct sunlight and perish below 9˚C or above 52˚C (Bundy and Cooper, 1989; Stephenson *et al*., 2000; CDC, 2013).

There is no direct person to person transmission, or direct infection from fresh feces, because the eggs require the right ambient conditions and time to embryonate and become infective (Bundy and Cooper, 1989). *T. trichiura* does not multiply in the human host (autoinfection), reinfection occurs only as a result of accidental ingestion of an embryonated egg (Bundy and Cooper, 1989).

Interestingly, in the mouse model, the close relationship between *Trichuris muris* and the composition of the gut microbiota has been explored in regards to egg hatching triggers and the successful establishment of infection (Hayes *et al*., 2010). Throughout its life cycle *T. trichiura* excrete and secrete a variety of molecules that interact with the host environment (Bancroft *et al*., 2019; Cruz *et al*., 2021). Some are antigenic and some are immunomodulatory, and the functions of many of them are still to be identified (Bancroft *et al*., 2019, Cruz *et al*., 2021).

## 1.2.3   Morphology of developmental stages

### 1.2.3.1   *Trichuris trichiura* egg anatomy

The lemon-shaped, bile-stained egg contains three layers. First, an outer vitelline layer derived from the fertilized oocyte's vitelline membrane. The vitelline layer develops the entire egg, including the bipolar plugs. After, a middle chitinous layer with high tensile strength adapted to protect the enclosed embryonic material and larva, and lastly, an inner lipid layer. The clear bipolar plugs are composed of the same three layers: vitelline, chitinous, and lipid, and are supported by

a "collar" composed of the middle chitinous layer that fans out from the shell (Appleton and White, 1990) (**Figure** 1.4).
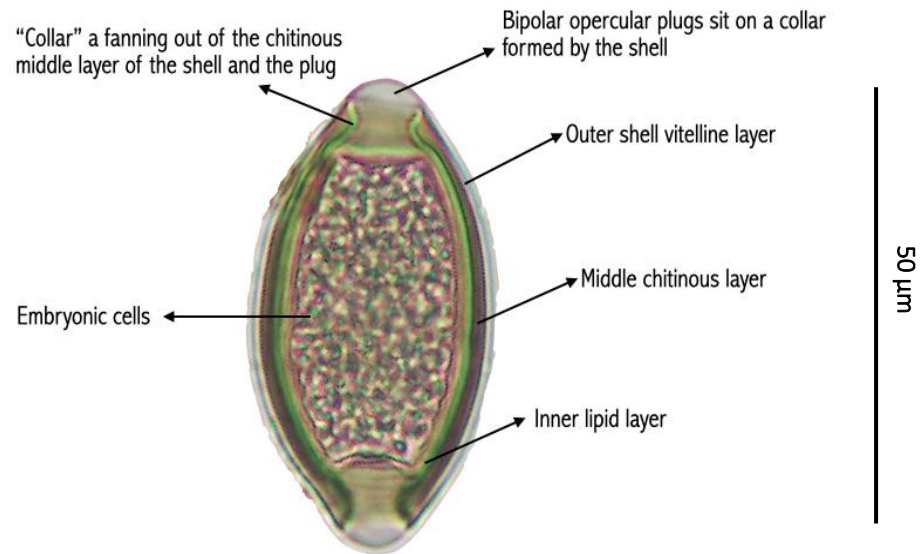


Figure 1.4 *Trichuris trichiura* egg anatomy.

### 1.2.3.2 *Trichuris trichiura* larval stages

The larval developmental stages, as described by Fahmy (1954), show the L1 (2) larvae recently released from the egg as early as 2 hrs and 30 min post-infection. Nine days post-infection (3), the L1 showing a more defined esophagus and intestine structures. The L2 larvae (4) after the first molt with distinct esophageal cells and the same L2 larvae in the process of molting for the second time (5) (**Figure** 1.5).
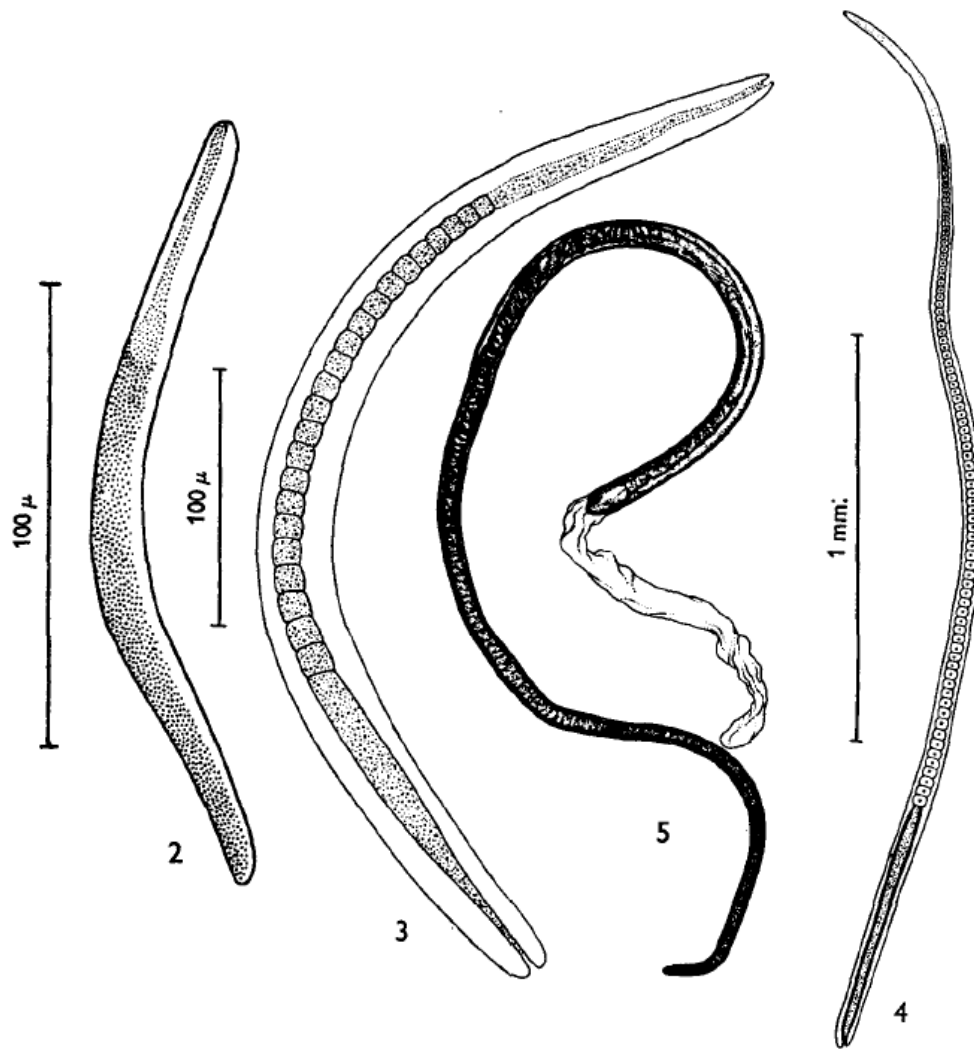
**Figure 1.5 Larval developmental stages**
(2) newly hatched larva (L1), (3) 9 days after infection, (4) at 17 days, (5) Larva in the process of molting
(23 days post-infection) (Adapted from *T. muris* in Fahmy (1954)).

After the second molt, the L3 larvae displays well developed reproductive organs. The spicule and testis can be identified in the male (6) with the characteristic coiled posterior and an overall smaller length. The long and thick posterior end with the vulva opening, the ovary, and the oviduct are identified in the slender and longer female (7) (**Figure** 1.6).
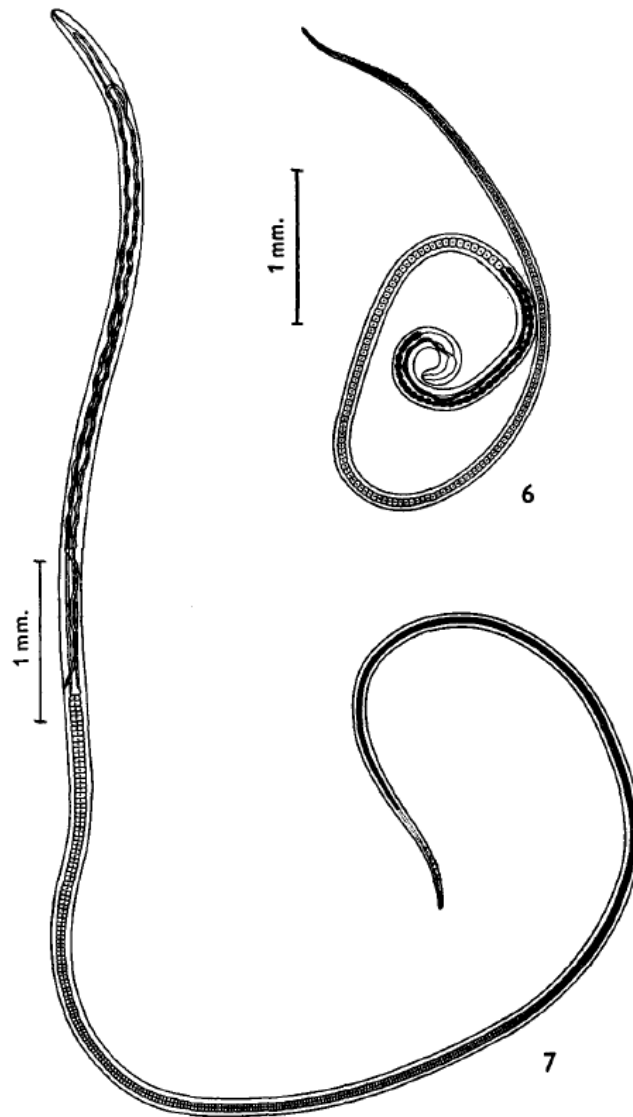
**Figure 1.6 Larval developmental stages (continuation)**
L3 Larvae 27 days post-infection (6) Male larva (7) Female larva (Adapted from *T. muris* in Fahmy (1954)).

### 1.2.3.3  *Trichuris trichiura* adult female and male

The fully developed adult trichuris, male and female, display a long and slender anterior portion and a wide and thick posterior, giving the whip's appearance. The male is smaller than the female, and in both, the esophagus extends two-thirds of the body length composed by a single layer of stichocytes (**Figure** 1.7).
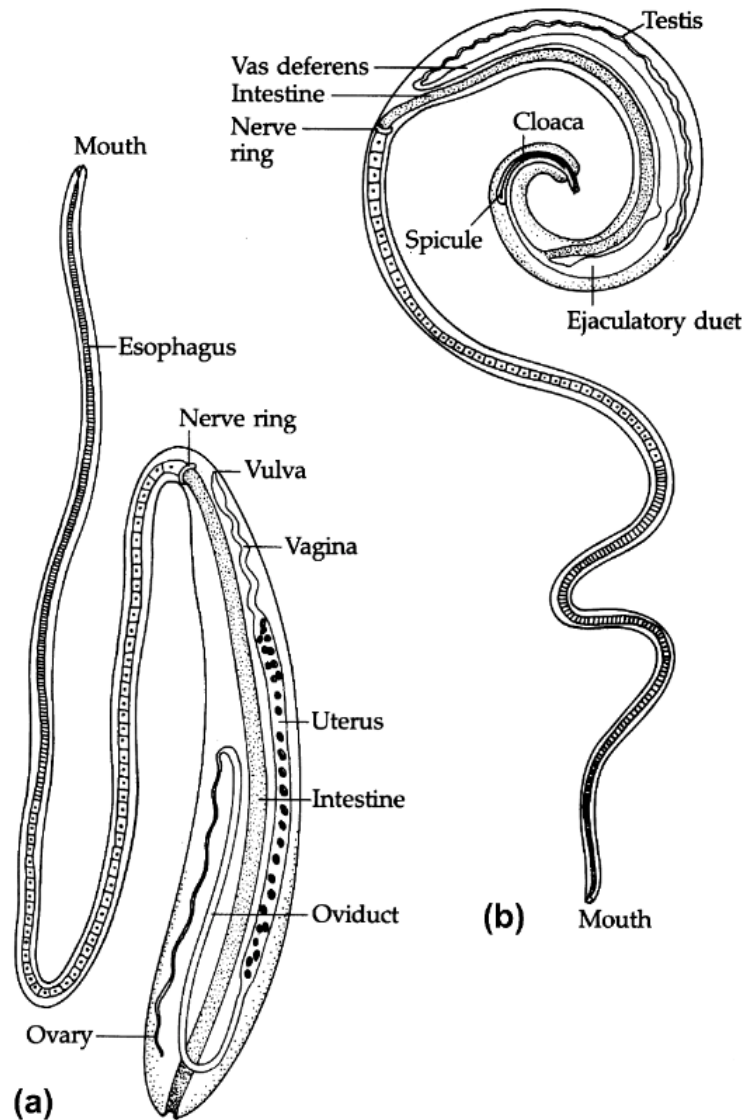
Figure 1.7 *Trichuris trichiura* adult anatomy
(A) female (B) male 32 post-infection (Adapted from Bogitsh *et al*. (2013))

### 1.2.4 Genomics and Proteomics

The understanding of *Trichuris trichiura* genome and proteome to date represent the basis of the work presented here. We have used them to identify gene families and respective processes associated with host invasion and drivers of *T. trichiura* infection.

The *Trichuris* genus has a wide geographical distribution which makes it a likely candidate to contain cryptic species, that is those that cannot be distinguished using conventional morphological examination methods (Betson *et al.*, 2015). Whipworms in humans are traditionally designated as *T. trichiura,* but the possibility of several species affecting humans and some of these being shared with nonhuman primates (NHPs) has been explored (Cavallero *et al.*, 2019; Di Filippo *et al.*, 2020). Hawash (2016) and Rivero (2021) described how genetic similarities between species found in humans and NHPs also suggests that species of *Trichuris* found in humans represent an heirloom parasite, meaning a parasite inherited from our primate ancestors.

The inference of the phylogenetic relationship between nematodes based on ITS sequences can be problematic, which is why it is recommended to supplement this type of analysis with other genetic markers such as mitochondrial DNA (mtDNA) genes (Callejón *et al.*, 2013). Sequencing of nuclear and mitochondrial markers from a range of wild and captive NHP host species has clarified this taxonomic relationship between worms and the potential zoonoses. Ravasi (2012) compared Internal Transcribed Spacers (ITS1-5.8S-ITS2) sequences from South Africa's adult *Trichuris* sp. from wild baboons and humans, finding a potential cross-infection as both *Trichuris* sequences shared the same two distinct clades. Ghai (2014) compared *Trichuris* full mtDNA genome from humans in China and Uganda to adult nematodes from captive baboons in the USA and Denmark, finding that there was more than one species involved in these infections based on the genetic distances observed. Cavallero (2015) integrated new phylogenetic results from *Macaca fuscata* and *Cercopithecus aethiops* with Ravasi (2012) and Callejón (2013) findings, providing further evidence of the distinct clades and subclades confirming the existence of additional separated taxa amongst NHP and human *Trichuris* species.

Cavallero (2019) presented the largest dataset to date (*rrn*L gene, the large subunit rRNA-encoding gene from the mitochondrial (mt) large subunit ribosomal RNA (rRNA)) and the most representative phylogenetic tree showing two main clades and of relevance to our work. The first,

named clade 1 CPGOB, grouped *Trichuris* species isolated from European zoological parks including nonhuman primate species (*Chlorocebus sabaeus, Chlorocebus aethiops, Colobus sp*.) and pig (**Figure** 1.8). The second clade, named clade 2, includes 4 branches: subclade Clade 2A (*Papio hamadryas*, *Macaca fuscata*, *Chlorocebus sabaeus* (St. Kitts isolate)*, Papio* sp. and humans); subclade MF (*M. fuscata*); subclade H (humans) and a sister branch of *Trichuris* infecting *Trachypitecus francoisi*. It is of note that all *Trichuris* sequences from the *rrn*L gene from the genus *Chlorocebus* clustered together in Clade 1, except for the isolates obtained from the monkeys studied in our current work. The Saint Kitts *C. sabaeus*, is clustered in Clade 2, subclade 2A (Cavallero *et al.*, 2019), suggestive of a *C. sabaeus* species that has developed isolated in the island of St. Kitts (**Figure** 1.8). Whipworms in humans, therefore, represent multiple *Trichuris* species, some of which we share with NHPs.
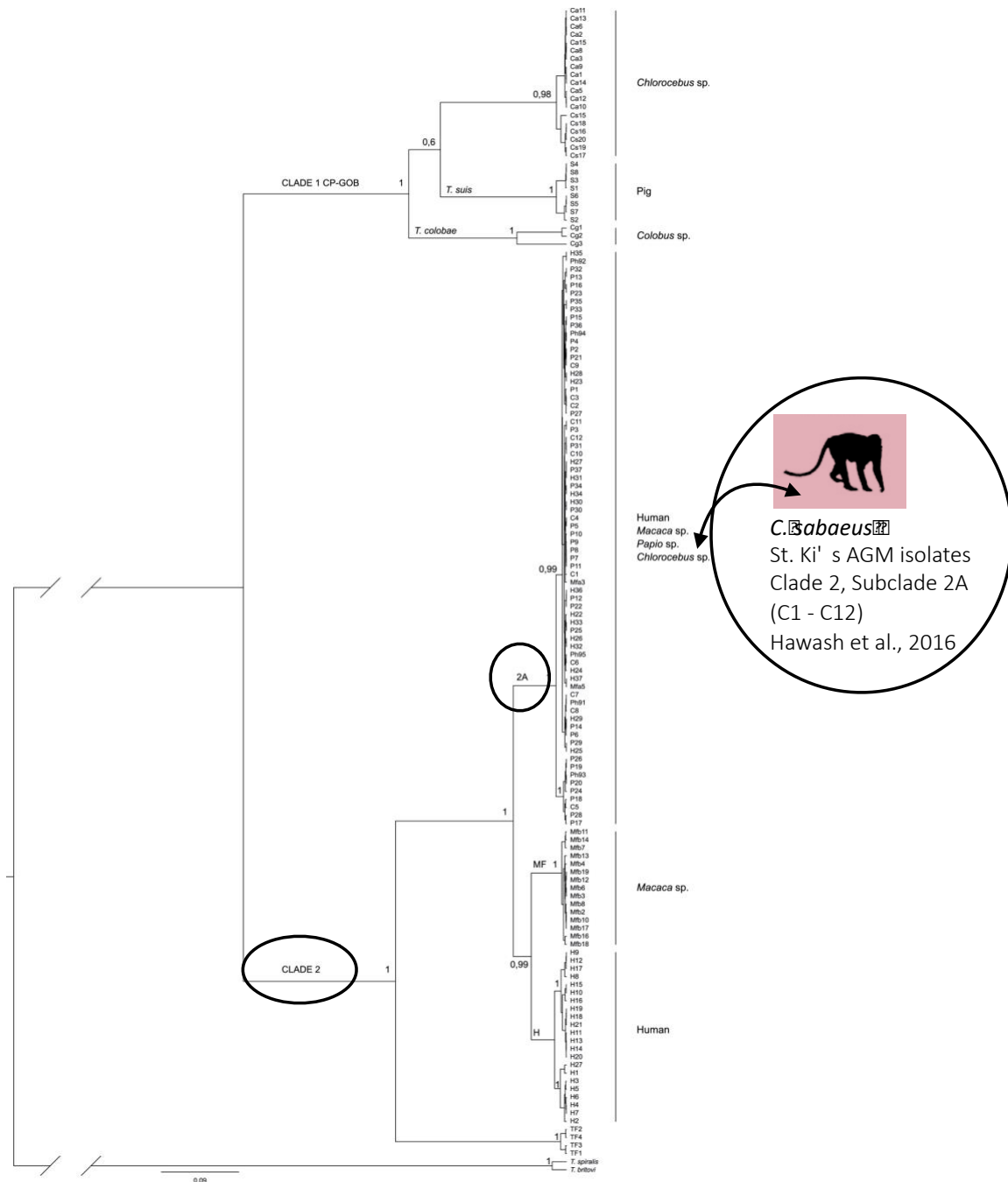
**Figure 1.8 Bayesian tree showing most up to date relationships among *Trichuris* species.**
Based on the analysis of the Dataset *rrn*L, including sequences used as outgroup (*Trichinella spiralis* and *T. britovi*), with indications on host affiliation and assignment to clades and subclades, following the nomenclature used in previous papers (Ravasi *et al*., 2012; Callejón *et al*., 2013; Cavallero *et al*., 2015). Adapted from Caballero *et al*. (2019).

In 2014, Foth and collaborators produced and published the draft genome assembly (75.2 Mb) from one adult male *T. trichiura* predicting, 9,650 genes, 2,350 of those genes being species-specific to *T. trichiura.* While this genome is considered to be 94.8% complete using Core Eukaryotic Genes Mapping Approach (CEGMA), the complete genome of the human whipworm is an ongoing project started in 2017 and led by the Wellcome Sanger Institute [PRJEB535](PRJEB535).

According to Foth (2014), the 2,350 species specific genes were dominated by hypothetical proteins of no known function. Specifically, a total of 2,014 hypothetical proteins were encoded by *T. trichiura,* with 253 (12.6%) proteins predicted to contain signal peptides, and 359 (17.8%) sequences predicted to contain transmembrane domains. These data represent a large number of proteins secreted and transported outside of the cell from which function remains unknown, including some of the proteins identified in our work.

From other sequences with known functional annotations extracted through the Gene Ontology (GO) term enrichment analysis, it was inferred that extracellular proteins, proteases, and protease inhibitors were particularly abundant among the remaining species-specific proteins (Foth *et al.*, 2014). In particular, serine proteases such as chymotrypsin A-like serine proteases were most abundant in both, gene number and gene expression (encoded by 75 genes) than other protease families, which is not the case in other nematodes such as *N. americanus* (Cantacessi *et al.*, 2010) and *S. stercoralis* (Marcilla *et al.*, 2010, Marcilla *et al.*, 2012). Therefore, chymotrypsin A-like serine proteases are the single largest group of proteases in the *T. trichiura* proteome and of relevance to the work presented here, because the stichosome and bacillary band in the anterior region are involved in the nutrient uptake, digestion, and host-parasite interactions (Foth *et al.*, 2014). Consequently, serine proteases could serve both; digestive and host-immunomodulatory functions as well as degrade host intestinal mucins that act as a physical barrier to the parasite (Foth *et al.*, 2014).

The *T. trichiura* genome also featured 20 genes encoding Whey Acidic Protein (WAP) domains, the Secretory Leukocyte Protease Inhibitor (SLPI)-like proteins, being the most abundant protease inhibitor transcripts in the anterior portion of the parasite, but surprisingly only one of them: TTRE_0000351901, in which the WAP domain contained the established eight cysteine residues recognized for this domain. The rest of the proteins containing the WAP domains present

a novel adaptation of six cysteine residues instead of eight, and the specifics of this modification in the function of the proteins remains unknown.

In addition to the protease inhibitor activity, SLPI-like proteins have anti-inflammatory, immunomodulatory, and antimicrobial properties on the host epithelial cells as it is invaded and wounded by the parasite, representing an important immunomodulatory function triggered in the innate immune defense and wound healing processes of the host (Wilkinson *et al*., 2011; Foth *et al*., 2014). It is of note that no other parasitic helminth lineage presents SPLI-like proteins, meaning that the corresponding genes are robustly and particularly expressed by the whipworm anterior portion (Foth *et al*., 2014). As described above, the majority of these proteins are secreted, suggesting that this family of proteins could carry a nematode clade I-specific function focused on inhibition of inflammation in the host response during invasion and establishment of the parasite (Foth *et al*., 2014), relevant to our findings for Kunitz BPTI domain containing protein.

Transcripts for DNAse-II-like proteins were also prevalently expressed in the whipworm anterior region (Foth *et al*., 2014). Normally, in other animals including *Caenorhabditis elegans*, free-living nematode, these proteins are involved in apoptotic DNA degradation and development (Lai *et al*., 2009), but the phylogenetic tree positioning observed by Foth (2014) leaves an open-ended question concerning the potential biological role of DNAse II-like proteins in *Trichuris* species.

The draft genome of *T. trichiura* also revealed male-specific expression of transcripts encoding proteins with Major Sperm Protein (MSP) domains, thought to have a role in the locomotion of the sperm (Tarr *et al*., 2005), and proteins with casein kinase-related and epidermal growth factor (EGF) like domains, these proteins are likely involved in male mating behaviors and functions (Hu *et al*., 2006). In contrast with male-specific proteins, transcripts for proteins with chitin-binding domains which are relevant to our findings for CBM 14 domain containing protein, were upregulated in the female whipworm. Nematode eggshells commonly contain chitin (Johnston *et al*., 2010); therefore, it is likely that these proteins are linked to the process of the egg formation (Foth *et al*., 2014).

Larvae L2 and L3 transcriptional landscape resembles that of the anterior region of the adult stages of the whipworm, likely due to the shared intraepithelial location and absence of the

reproductive and sex-specific transcripts that dominate the posterior portions of the adult *T. trichiura* (Foth *et al*., 2014). Also, larval stages had high expression of transcripts for ribosomal, collagen and fibronectin related proteins; most likely associated with the protein and cuticle synthesis necessary for the fast growth that these life stages undergo during their development (approximately 5,000-fold increase in body/muscle volume over a 35-day period) (Foth *et al*., 2014; Bancroft *et al*., 2019). These findings are relevant to polycysteine and histidine protein the most abundant found in our work.

The majority of *T. trichiura* genes are orthologs shared by both *T. muris* and *T. trichiura*, with 5,060 one-to-one protein orthologs presenting an average amino acid identity of 76% (Foth *et al*., 2014).

## 1.3    Trichuriasis

### 1.3.1    Epidemiology of the disease and population at risk

*Trichuris trichiura* infections favor the warm and moist conditions in tropical and subtropical regions where the appropriate temperature and humidity allow the life cycle of the soil-transmitted nematode to continue (Bundy and Cooper, 1989; Else *et al*., 2020). Transmission requires the excreted unembryonated and noninfectious eggs to embryonate and become infectious under ideal environmental conditions (Bundy and Cooper, 1989; Else *et al*., 2020), this fundamental aspect of its biology determines a set of transmission dynamics that are different from other human nematodes (Hotez *et al*., 2008). Consequently, temperature, humidity, and population density geographical information system tools have been used to enable prediction and estimation of the geographical distribution of parasite (Else *et al*., 2020).

Trichuriasis is considered a disease of the poor, where lack of education and sanitation infrastructure favors transmission. In these environments, the prevalence of infection, determined by the intensity of infection measured by Eggs Per Gram of feces (EPG) found in a single fecal sample, can be 90% and mainly affecting children of school age (5-15 years old) (Stephenson *et al.*, 2000; Else *et al.,* 2020). Historical data shows that the prevalence peaks earlier in life due to age-related risk exposure, and there is a decline in prevalence observed after school age and in

adults with lower risk exposure and potential age-acquired immunity (Stephenson *et al.*, 2000; Else *et al.*, 2020). Morbidity is associated with the number of worms infecting the host rather than the presence or absence of infection (Hotez *et al.*, 2008).

Epidemiological characteristics surrounding the parasitized patient such as age, sex, type of job, contact with soil, soil temperature, the consumption of potential contaminated food or water, weather conditions, and housing type, are essential when describing the population at risk (Mutombo *et al.*, 2019). Three main categories determine an increased risk factor (Stephenson *et al.*, 2000, Hotez *et al*, 2008; Sturrock *et al.*, 2017)

a. Environmental risk factors such as temperature, precipitation, soil conditions, and humidity.

b. Human socioeconomic risk conditions such as water access, sanitation, and population density.

c. Human behavior risk factors such as food preparation, hand washing, and hygiene education and geophagy.

Therefore, improvement in the above categories has demonstrated to be vital for the elimination and control of Soil-Transmitted Helminths (STHs), such as the case of industrialized countries (Lustigman *et al.*, 2012).

## 1.3.2 Clinical disease in humans and nonhuman primates

### 1.3.2.1 Humans

Clinical disease is caused by the inflammation of the cecum and the large intestine, due to the adult worm induced local inflammatory response and blood loss (estimated to be 0.005 mL per worm per day) (Else *et al.*, 2020). The clinical signs in humans are related to worm burden (Else *et al.*, 2020). In human infections, low-level infections (< 15 worms) are often asymptomatic, but moderate to high infections (hundreds of worms) can cause a range of symptoms including intestinal discomfort with abdominal pain, colitis, vomiting, and bloody diarrhea (Stephenson *et al.*, 2000; Else *et al.*, 2020). Infections in children can result in severe anemia, general malaise and weakness, stunted growth, and poor school performance (Stephenson *et al.*, 2000; Else *et al.*,

2020). Infections of very high intensity can cause intestinal obstruction that can require surgical interventions to avoid rectal prolapse (WHO, 2018). Sequalae nutritional impairment includes loss of iron, protein and an overall reduction of nutritional intake that can cause dysentery (Stephenson *et al*., 2000; Else *et al*., 2020).

*Trichuris* Dysentery Syndrome (TDS) results from very high intensity infections (several hundreds to thousands of worms) (Cooper *et al*., 1986; Stephenson *et al*., 2000). This severe illness, also known as massive infantile trichuriasis, is associated with iron deficiency anemia, chronic mucoid diarrhea, rectal bleeding, rectal prolapse, consequence of increased straining and/or peristalsis, and finger clubbing (Khuroo *et al*., 2010; Kaminsky *et al*., 2015). In adults however, TDS has been identified recently to be the causative agent of severe iron deficiency anemia, reflecting the poor clinical recognition of trichuriasis in general but mostly in communities with severe poverty, not included in MDA programs (Khuroo *et al*., 2010; Else *et al*., 2020). Colonoscopy reports from patients with this diagnosis revealed associated mucosal changes including, edema, petechial lesions, blotchy mucosal hemorrhages, active mucosal oozing, and luminal narrowing (Khuroo *et al*., 2010; Wang *et al*., 2013).

The quality of life is the most affected by chronic infections, contributing to the long term nutritional morbidity and affecting the cognitive development of infected children (Else *et al*., 2020).

### 1.3.2.2   Nonhuman primates

The clinical disease of nonhuman primates has not been studied in depth despite *Trichuris* being a highly prevalent parasite species amongst captive and wild populations (Abee *et al*., 2012; Calle and Joslin, 2014); therefore, human *Trichuris* literature is commonly used as guidance. Whipworms are the most common nematodes found in AGMs in previous reports, and they mainly focused on the description of treatment outcomes (Munene *et al*., 1998; Muriuki *et al*., 1998; Mutani *et al*., 2003; Gillespie *et al.,* 2004., Legesse *et al*., 2004; Petrášová *et al*., 2010; Gaetano *et al*., 2014; Kooriyama *et al*., 2014; Amenu et al., 2015; Li *et al*., 2015; Wren *et al*., 2015; Dalimi *et al*., 2016; Valenta *et al*., 2017; Teklemariam *et al*., 2018 and Barbosa *et al*., 2020).

*Trichuris trichiura* is the species generally thought to be present in AGMs, and this has been confirmed to be the species infecting *C. sabaeus* on St. Kitts (Hawash *et al.*, 2016; Yao *et al.*, 2018). While light infections in NHPs are often subclinical, the heavy infection produces severe enteritis with non-responsive gray mucoid diarrhea and inappetence, which have been reported in fatal *trichuris* infections with hemorrhagic typhlitis and ileal intussusception found at necropsy (Hennessy *et al.*, 1994; Emikpe *et al.*, 2002; Eo *et al.*, 2018). In addition, chronic infections have been reported to exacerbate hepatopathology caused by *Schistosoma mansoni* and a higher female worm burden (Le *et al.*, 2020) and have been associated with behavioral shifts (Chapman *et al.*, 2016) where the AGMs had reduced rates of movement and socialization.

### 1.3.3 Diagnostics

#### 1.3.3.1 Parasitological methods

Diagnosis of trichuriasis relies on the examination of a fecal sample in order to determine presence or absence of eggs and, if possible, a quantification of the eggs present in the sample analyzed. To date, the WHO recommends using the Kato-Katz and direct microscopy fecal examination method including a two slide preparations per sample analyzed (WHO, 2018).

Other parasitological diagnostic methods (**Table** 1.2) include formol-ether concentration, FLOTAC, mini-FLOTAC, McMaster and double centrifugation with Sheather's sugar solution, but the sensitivity of these methods depends directly on the variability of the infection intensity experienced by the host (Nikolay *et al.*, 2014; Else *et al.*, 2020).

Molecular-based parasitological diagnostic methods, with superior sensitivity, have been developed in an effort to counter the overall reduced sensitivity of microscopy-based parasitological methods (Else *et al.*, 2020; Fernández-Soto *et al.*, 2020). However, the requirement of specific and more expensive equipment and DNA extraction from stool samples limits the application in field settings and endemic areas with limited resources. The Loop-Mediated Isothermal Amplification (LAMP) test is a species-specific colorimetric isothermal assay (between 59 °C– 66 °C) that offers an alternative to traditional PCR in field conditions and low-income areas providing accessible, cost-effective, easy-to-perform method (Ngari *et al.*, 2020). Also, LAMP test

has shown to identify infections more than 15 days before the initial identification of eggs in the feces, and it is also applicable to urine samples (Fernández-Soto *et al.*, 2020). The ability to identify infections during the prepatent period of the parasite (that is, the time of infection to egg production identified in the feces) poses the biggest challenge to trichuriasis diagnosis to date (Cruz *et al.*, 2021).

Table 1.2 Parasitological diagnostic methods for *Trichuris trichiura.*

| Test | Output | Sensitivity % | Specificity % | Advantages | Limitations |
|---|---|---|---|---|---|
| Direct microscopy | Egg detection | 62.8 | 97.5 | Low cost | Positive results only at high parasitic burden |
| Kato–Katz | Egg detection and egg quantification | 62.8–91.0 | 97.5 | Relatively low cost; possible to determine the burden of infection | Overall low sensitivity (Especially at light infection intensities). Need for a qualified microscopist. |
| Formol–ether | Egg detection | 81.2 | 97.5 | Relatively low cost | |
| FLOTAC | Egg detection and egg quantification | 88.7–100 | 97.5 | Detection of different STHs simultaneously | Requires centrifugation steps with two different rotors; relatively high cost |
| Mini- FLOTAC | Egg quantification | 76.2–91.5 | 97.5 | Detection of different STHs simultaneously | Limited sensitivity (Sensitivity comparable to that of the Kato–Katz method but lower than that of FLOTAC) |
| FECPAK | Egg quantification | 59.8–65.8 | 97.5 | Detection of different STHs simultaneously. simple procedure with results within an hour | Requires internet connection |
| Double centrifugation with Sheather's sugar solution | Egg quantification | No data in humans | No data in humans | Relatively low cost. Detection of different STHs simultaneously, less common in human diagnostics. Widely used in Veterinary Medicine. | Requires two centrifugations steps; sugar solution can create sticky surfaces. |
| McMaster | Egg quantification | 61.1–81.8 | 97.5 | Possible to detect multiple infections by multiplexed assays; high specificity | Need for a special counting chamber |
| LAMP assay | Identification of DNA from *Trichuris* spp. | 100 | 100 | Possible to detect multiple infections by multiplexed assays; high specificity | Risk of low sensitivity due to the presence of inhibitors in the fecal sample; decreased sensitivity if formalin is used for fixation of samples, requires specialized equipment, and has restricted use in the field |
| qPCR | Quantification of DNA from *Trichuris* spp. | 72.3–100 | 100 | | |

Adapted from Nikolay *et al*. (2014) and Else *et al.* (2020).

### 1.3.3.2 Immunological diagnostic methods

The focus of immunological diagnostic methods to date is on research and discovery, to uncover the molecules excreted, secreted, or contained within all the developmental stages of *T. trichiura.* These parasite-derived molecules interact with the host environment during the prepatent period of the parasite, before the eggs appear in the feces (Bancroft *et al.*, 2019; Cruz *et al.*, 2021). The immunological diagnostic approach would allow the early diagnosis of trichuriasis and diagnostic methods that do not rely on inconsistent clinical signs or fecal analysis to detect infections (Else *et al.*, 2020; Cruz *et al.*, 2021). Novel immunological diagnostics are crucial to detect infections following accidental ingestion and during the prolonged prepatent period that can last anywhere between 8 to 16 weeks (Bundy and Cooper, 1989; Hansen *et al.*, 2016)

There are no commercially approved antibody detection tests for the diagnosis of *T. trichiura* (Mutombo *et al.*, 2019; Else *et al.*, 2020). The association between *T. trichiura* infections and anti-*Trichuris* salivary IgG was explored by Needham (1996) and collaborators in the 90's. Their findings using saliva samples and crude antigen extract for their indirect ELISA assay, showed a correlation between age and infection intensity allowing for diagnosis at the community level, but not individual diagnosis.

Challenges on antibody detection assays remain in the differentiation of active and past infections as well as their ability to correlate with worm burden (Mutombo *et al.*, 2019).

### 1.3.4 Treatments

### 1.3.4.1 Humans

The WHO recommendations include preventive chemotherapy with the implementation of a biannual single-dose of albendazole (400 mg) or mebendazole (500 mg) when the baseline prevalence is over 50%, and annual therapy when the baseline prevalence is lower than 50% (WHO, 2017). This regimen is recommended as a public health intervention for children 2 to 12 years old, for all adolescent girls 10 to 19 years old, and non-pregnant women of reproductive age (15 - 49 years old). The same regimen is also applied for pregnant women after the first trimester in areas of high STHs prevalence (WHO, 2017). These regimens have shown efficacy (based on Egg

Reduction Rates (ERR)) of 99.9% for *A. lumbricoides*, 92.4% for hookworms, and 64.4% for *T. trichiura* (WHO, 2013), and are also followed during MDA campaigns without a previous individual diagnosis to all the at-risk groups to reduce morbidity, worm burden, and help maintain a low intensity of infection (WHO, 2018).

### 1.3.4.2  Nonhuman primates

*Chlorocebus sabaeus*, African green monkeys (AGMs) of the Cercopithecidae family, widely found in Africa, were introduced onto the Caribbean island of St. Kitts in the 17th century (McGuire., 1974). There is little data on the parasites of the vast feral population of AGMs (perhaps 50,000) (Dore *et al*. 2021), which commonly come into contact with people and may play a role in the epidemiology of human zoonotic parasitic infections common on the island (Berger, 2020). Incomplete Caribbean studies have reported island AGMs to be infected with *Trichuris trichiura* (Yao *et al.* 2018), *Strongyloides* spp. (Ritchie *et al.* 1967; Gallagher *et al.* 2019), *Primasubulura* sp. (Cameron, 1930) and *Schistosoma mansoni* (Cameron, 1928).

There is limited data on anthelmintic treatments for AGMs and husbandry recommendations to control parasitic infections in captive research animals. Research facilities require effective parasite treatment and prevention protocols to minimize infections that may confound research outcomes and cause unnecessary stress, which lowers the quality of life of study animals. Ritchie *et al.* (1967) reported that thiabendazole (two 50 mg/kg doses PO 14 days apart) significantly reduced or eliminated *Strongyloides* spp. eggs in the feces. Kagira et al. (2011), reported that a three-day regimen of albendazole (7 mg/kg, PO) and ivermectin (300 µg/kg, SQ) reduced the Fecal Egg Counts (FECs) of *T. trichiura* and strongyles by 100% consistently after 7, 14 and 28 days. Chapman *et al.* (2016) reported that two doses of ivermectin (300 µg/kg, PO) five days apart resulted in a 100% cure rate for nematodes in wild AGMs, with no eggs found in the feces 1-month post-treatment.

Unfortunately, evaluation of nematode infections is challenging because of inadequate data on drug efficacy in AGMs and the lack of studies with prolonged follow-up which is needed to detect confounding factors (autoinfection, reinfection, larval transplacental or colostrum transmission, dormant stages, and autoinfection due to parthenogenesis) (Reichard *et al.,* 2017).

Also, FECs can be unreliable, and studies that enable necropsy evaluations are relevant in the absence of a 'gold standard' (Geary *et al*., 2010).

## 1.4　Host immune response against *Trichuris trichiura* infection

Eggs contain the first antigens of *T. trichiur*a that are presented to a naïve host's immune system and would thus seem to be the most important in the development of an early and effective immune response to limit infection (Cruz *et al*., 2021). Also, once the adult worms are tethered to the intestinal mucosa, they secrete and excrete an abundance of proteins that have been shown to elicit protective immune responses (Lillywhite *et al*., 1995; Santos *et al*., 2013) and immunomodulate the host immune response to facilitate their parasitism in the host (Foth *et al*., 2014).

Bancroft and collaborators (2019) described how the major secreted protein of *T. muris,* p43, also the most abundant in our work presented here for *T. trichiura,* PCHTP-2, (Cruz *et al*, 2021), is able to bind the host IL-13 and separate the cytokine within the host extracellular matrix, preventing it from activating the type 2 immune response.

There is however, limited available information of $T_H1$ and $T_H2$ immune responses from humans and nonhuman primates infected solely with *T. trichiura*, as polyparasitism is the most usual presentation of the disease (Else *et al*., 2020). Nevertheless, it is strongly inferred that *T. trichiura* induces a type 2 immunity response and that acquired immunity requires type 2 protective immune responses that develops slowly after prolonged exposure (Else *et al*., 2020).

It is also understood that epithelial cells on the host frequently produce SPLIs proteins when responding to inflammation in order to modulate the inflammatory process, the cytokine secretion, and the cell recruitment and to favor $T_H1$-type immune response (Foth *et al*., 2014). Interestingly as described in section 1.2.4., this family of proteins appears to carry a nematode clade I-specific function that inhibits the inflammatory responses on the host during invasion and establishment of the parasite (Foth *et al*., 2014).

Furthermore, in recent years the role of Extracellular Vesicles (EVs) in *Trichuris* spp. infection has been described. Extracellular vesicles are a population of lipid-enclosed spherical vesicles that participate in the communication between cells and organisms (Mardahl *et al*., 2019).

They are released by cells into the extracellular space and contain bioactive components (Mardahl *et al.*, 2019). In 2015, it was described for the first time that EVs were released from whipworms and that L1 larvae of *T. suis* secret small RNAs apparently involved in host-parasite interactions (Hansen *et al.,* 2015). In 2018, Shears and collaborators described how fractions of excretion/secretion (ES) products of *T. muris* elicited long-lasting immunity against reinfection, showing that EVs induced a mixed $T_H1$ and $T_H2$ response.

Challenges including genetic heterogenicity, undefined infection history, exposure intensities, and co-infections continue to blur the clear picture of *T. trichiura* host immune responses (Else *et al.*, 2020; Hayon *et al.*, 2021).
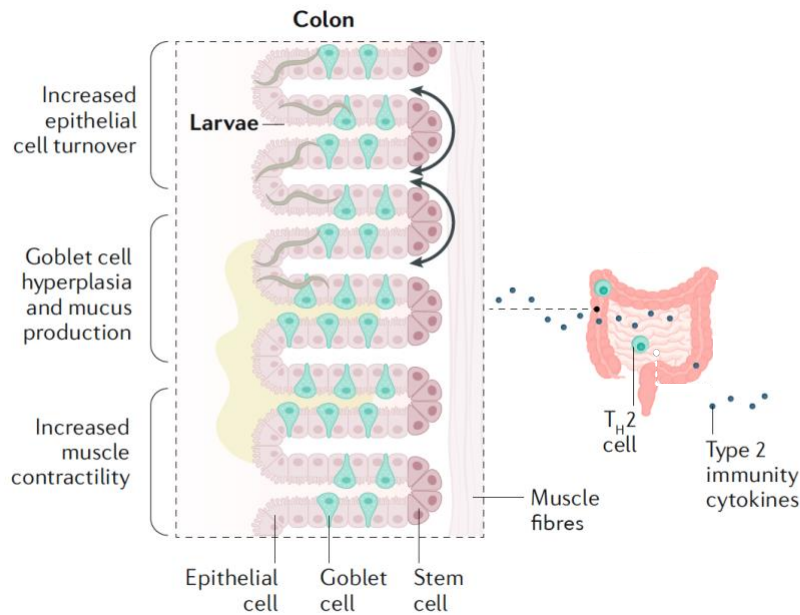


**Figure 1.9 Type 2 immunity**
Type 2 immunity cytokines (IL-4, IL-5, IL-9, and IL-13) increase the rate of epithelial turnover, the goblet cell numbers, and mucus production, and increase in muscle contractility resulting in epithelial turnover and parasite expulsion (Adapted from Else *et al.*, 2020).

## 1.4.1  Antibody Response

Although antibody response to *T. trichiura* has not been studied in-depth due to limitations inherited from the lack of an adequate animal model and the difficulties obtaining the parasite

material (Else *et al*., 2020), Lillywhite (1991) and collaborators showed that infection with *T. trichiura* evokes a strong humoral immune response of serum IgG, IgA, and IgE classes.

Lillywhite (1991) found that the predominant humoral response was mediated by IgG, showing a correlation to infection intensity that was later confirmed by Needham (1994). Serum IgE antibody responses also studied by Needham (1994) were difficult to interpret as the antibody signals were negligible to the crude antigen extract used. Also, serum IgA antibodies showed a vigorous unspecific response during early childhood, and adulthood, in low and high transmission areas suggesting a potential association with acquired immunity to *T. trichiura* (Needham *et al*., 1994).

Further, Needham (1996) investigated the salivary antibody responses based on the concept of a common mucosal immune system where antibodies secreted in the saliva, and other mucosal secretions would be expected to reflect the same gut-associated antigen responses. The salivary IgA levels showed a weak but significant negative correlation with infection intensity and an increase with age, suggesting the confirmation of its association with acquired immunity (Needham *et al*., 1996).

Faulkner and collaborators (2002) showed that serum IgE levels were strongly associated with an age-dependent decrease in *T. trichiura* infection intensity (the older and less heavily infected study subjects had greater levels of serum IgE), while serum IgG and IgA were inconsistent in mirroring infection intensity and age correlation.

Self-infection studies showed that serum IgA and IgG anti-*Trichuris* antibody levels remained below detection levels before *T. trichiura* infection and peaked at the time of the establishment of infection while remaining above the detection levels for the remaining of the study inclusive of the post-treatment period (Dige *et al*., 2016).

Of note, the few studies described above that have studied *T. trichiura* antibody responses to date used crude antigen preparations because the nature and functional characteristics of *T. trichiura* adults, larval and egg life stages were largely unknown. Not until 2014, Foth and collaborators published the first draft genome for *T. trichiura,* while the complete human *T. trichiura* genome project is still an ongoing effort.

### 1.4.2   T helper lymphocytes

The T helper 2 ($T_H2$) or T helper 1 ($T_H1$) immune response in the host determines the possibility to resist ($T_H2$) or be susceptible ($T_H1$) to *Trichuris* infection (Hayon *et al.*, 2021). Based on animal models, $T_H2$ cytokines are associated with resistance to infection and the mechanisms needed to trigger the immune-mediated parasite expulsion from the host (Hayon *et al.*, 2021). Cytokines IL-4, IL-5, IL-9, and IL-13 have been represented as the promoters of *Trichuris* control inducing gut hypercontractility (IL-9 - recently moved from $T_H2$ to $T_H9$), increasing mucus production, and promoting epithelial turnover (IL-13) for the rapid expulsion of the parasites (Hayon *et al.*, 2021). For example, IL-4 receptor blockage polarized to a $T_H1$ immune response and promotes chronic infection, whereas administration of IL-4 to susceptible mouse strains results in a predominant $T_H2$ response and clearance of infection, defining IL4 as a critical mediator to enhance antibody responses (Briggs *et al.*, 2018; Hayon *et al.*, 2021). In contrast, during chronic trichuriasis (low dose prolonged infections), high concentrations of typical $T_H1$ immune response interferon γ (IFN-γ), IL-12, and IL-18 have been identified, where IL-18 drives suppression of IL-4 and IL-13 $T_H2$ associated cytokines, reducing rapid parasite expulsion, and making the host more susceptible to persistent infection (Shears *et al.*, 2018; Hayon *et al.*, 2021). Furthermore, when IFN-γ is depleted a reduction in IL-18 creates a more resistant host (Hayon *et al.*, 2021).

There is no analysis of type 1 and type 2 cytokines released by peripheral blood leukocytes and re-stimulated in vitro for *T. trichiura* because polyparasitism is the most common presentation in endemic populations where studies have been conducted (Else *et al.*, 2020; Hayon *et al.*, 2021). The challenges of understanding the role of T helper lymphocytes for isolated *T. trichiura* infections have been only recently elucidated with single-subject self-infection studies (Hansen *et al.*, 2016; Dige *et al.*, 2017). The longitudinal analysis of T cell subsets in mucosal biopsy samples and peripheral blood revealed a mixed mucosal T cell response ($T_H1$, $T_H2$, $T_H17$, and regulatory T ($T_{reg}$) cells) while circulating T helper cells became predominantly $T_H2$ cells (Hansen *et al.*, 2016; Dige *et al.*, 2017). In the *T. muris* model, high levels of local inflammatory $T_H17$-dependent IL-23 expression have been observed, highlighting the importance of its role for an accelerated worm expulsion. (Gomez-Samblas *et al.*, 2017).

There is an urgency to develop a vaccine against human whipworm (Hayon *et al.*, 2021). Significant efforts have led to discovering potential vaccine candidates; however, understanding the protective immunity of these candidates relies on preclinical models, like the murine model, which has led to a lack of consensus on the ideal development strategy (Hayon *et al.*, 2021). The challenges encountered with the murine model are the selection of the mouse strain to balance the host susceptibility to infection and how different *T. muris* strains or isolates can affect the success of the model (Hayon *et al.*, 2021).

### 1.4.3   Co-infections

Co-infections often affect children and are generally underdiagnosed as they remain associated with non-specific gastrointestinal symptoms (Else *et al.*, 2020). In endemic areas, chronic infections and misdiagnosis can lead to older adults experiencing Chronic Deficiency Anemia (CIDA) with hemoglobin levels less than 8 g/dL, iron deficiency, and TDS diagnosis upon confirmation of *Trichuris* infection (Khuroo *et al.*, 2010).

Co-infections in captive populations of AGMs are also common (Cruz *et al.*, 2021). They can remain undiagnosed as they can be associated with non-specific gastrointestinal symptoms that AGMs easily mask confounding daily observations and diagnoses (Cruz *et al.*, 2021). In the wild, naturally occurring co-infections are commonly described and understudied as most reports focus on the description of the treatment outcomes (Munene *et al.*, 1998; Muriuki *et al.*, 1998; Gillespie *et al.,* 2004., Legesse *et al.*, 2004; Petrášová *et al.*, 2010; Gaetano *et al.*, 2014; Kooriyama *et al.*, 2014; Amenu et al., 2015; Wren *et al.*, 2015; Dalimi *et al.*, 2016; Valenta *et al.*, 2017; Teklemariam *et al.*, 2018).

## 2 Justification and Objectives

Trichuriasis represents a public health threat. Concerns on treatment efficacy, low cure rates, drug resistance, and chronic illness compromise the control and eradication efforts. Under-diagnosis and lack of understanding of potential zoonotic exposures due to human encroachment and wildlife interactions expose the urgent need for a suitable animal model that enables access to parasitic material (feces, adult nematodes) and biological samples (serum and saliva) that facilitate the study of *T. trichiura* alternative diagnostic methods.

### 2.1 General Objective

Identify *Trichuris trichiura* immunogenic proteins of diagnostic value and evaluate anthelmintic treatments for captive AGMs for their application in animal and human trichuriasis prevention and control programs.

### 2.2 Specific Objectives

1. Evaluate the use of albendazole and ivermectin as anthelmintic treatment for AGMs and describe the husbandry recommendations used to control *T. trichiura* infections in captive research animals.
2. Analyze the correlation between fecal examination egg counts results and total worm counts found post-treatment in the large intestine.
3. Characterize and describe the *T. trichiura* egg proteome.
4. Identify *T. trichiura* immunogenic proteins of diagnostic value and lay out the process of designing and selecting synthetic peptides for diagnostic use.
5. Study the humoral immune response in saliva and serum to monitor the treatment outcomes in naturally infected AGM.

# 3 Materials and Methods

## 3.1 Ethical statements

The Institutional Animal Care and Usage Committee (IACUC) of the St. Kitts Biomedical Research Foundation (SKBRF) & Virscio Inc. (IACUC AC1875, Study code S00147) approved the collection of saliva and serum samples. The IACUC of Ross University School of Veterinary Medicine (RUSVM) approved postmortem tissue and sample collection from African green monkeys (2018 - Immunodiagnostics for *Trichuris trichiura,* P. Kelly).

Use of existing anonymous human saliva, serum and plasma samples from healthy volunteers were approved for use under RUSVM Institutional Review Board (IRB) exemption certifications No. 18 -11 EX and No. 18 -10 EX.

## 3.2 *Trichuris trichiura*

### 3.2.1 Adults

Adult worms were obtained at routine necropsy of naturally infected *C. sabaeus* that were humanely euthanized as part of other studies conducted at the SKBRF facility on St. Kitts. The abdominal cavity was opened, and the intestines ligated at the proximal duodenum and distal rectum before they were removed and placed in 0.9% saline for transport to the RUSVM Research Laboratory. Upon arrival, 4 grams of fecal material was extracted from the rectum for analysis and the large intestine separated from the small intestine. After the large intestine was opened longitudinally, a 2x2 cm full thickness section was removed from cecum and placed in 10% formalin for histopathology. The cecal samples were dehydrated in a series of ethanol solutions (30%, 50%, 70%, 80%, 90%, 95% and 100%) they were embedded in paraffin wax (BMJ-III embedding machine, Jiangsu, China) at 56 °C and sectioned. The 5 µm sections were stained with hematoxylin-and-eosin (H&E) and examined with an Olympus DP73 microscope (Tokyo, Japan) for morphological and structural changes. The remaining large intestine was soaked in saline solution (0.9%) for 2 hours at room temperature (30-32 °C) to loosen the intestinal contents and subsequently at 4 ˚C until analysis. Within 24h, any ingesta remaining attached to the mucosa was removed manually into the saline solution and the surface visually inspected for *T. trichiura* that remained attached.

The solution of the collected ingesta was filtered through a 100 µm sieve and the recovered material examined for adult nematodes under a stereomicroscope (7x – 10x magnification). The *T. trichiura* identified were counted, sexed, and washed five times for 5 min in Phosphate Buffered Saline (PBS; pH 7.4) before they were dried and preserved at -80 ˚C or, in the case of the most intact specimens, placed in Karnovsky's fixative (**Appendix I**) for Scanning Electron Microscopy. Other species of nematodes found were preserved in either 10% formalin or 70% ethanol for future morphological and molecular characterization.

### 3.2.2   Adults extracts (AE)

Adult females (n=10) and males (n=10) stored at -80 °C were defrosted at 4 °C, pooled by sex, and placed in PBS containing 1% protease inhibitors (cOmplete™ mini EDTA-free tablets, Roche, Mannheim, Germany) and 1% Triton™ x-100 (Sigma-Aldrich®, MO, USA). Five pulses, 30 s each, of a low speed mechanical rotor (Dremel®, Robert Bosh GMBH, Mount Prospect, IL, USA) was used to initially break down the worms which were maintained on ice during the procedure. After freezing at -20 °C and thawing at room temperature the nematodes were sonicated on ice with a Microson Ultrasonic Cell Disruptor XL™ (Misonix, NY, USA) using ten cycles of 10x 1-second pulses at maximum intensity. Following centrifugation (10,000 x *g* for 10 min at 4 ˚C) the supernatant containing the soluble proteins (the Adult Extract - AE) was removed and stored at -20 ˚C.

For the specificity assays, protein extracts from other species of helminths (see **Table** 3.1) were obtained, as above, from convenience samples of the following species:

Table 3.1 Convenience samples used to prepare protein extracts for the specificity assays.

| Sample species | Source |
|---|---|
| *Fasciola hepatica* and *Dicrocoelium dendriticum* | adults were obtained from infected cow's livers from an abattoir in Valencia (Martínez Loriente S. A., Valencia, Spain) in collaboration with Dr. Fernando Cantalapiedra. |
| *Aspiculuris tetraptera* | adults from mice were donated from concurrent studies at the Parasitology Department of University of Valencia. |
| *Anisakis simplex* | adults were obtained from whole cod bought commercially and examined in the parasitology laboratory as part of other projects. |
| *Gnathosoma hispidium* | adults were obtained from infected cows from the local abattoir in Valencia (Martínez Loriente S. A., Valencia, Spain) in collaboration with Dr. Fernando Cantalapiedra. |
| *Primasubulura* sp. | as described for *T. trichiura*, adults were obtained from ceca of AGMs at necropsy. |

### 3.2.3  *Trichuris trichiura* non-embryonated (NE) egg extracts (EE)

The uteri were dissected and extracted (**Figure** 3.1 (A) and (B)) from *T. trichiura* adult females (n=50) thawed from -80 °C at room temperature using a 30G ½" needle (BD Microlance™, Fraga, Huesca, Spain) (10x – 30x magnification) and placed in PBS. After, the uteri were opened with a longitudinal incision to facilitate the release of non-embryonated (NE) eggs which were pooled. After five washes in 1 mL PBS (10,000 x g; 1 min) the supernatant was removed and the NE resuspended in 700 μL PBS containing 1% protease inhibitors (cOmplete™ mini EDTA-free, Roche®) and 1% Triton™ x-100 (Sigma-Aldrich®). To ensure disruption of *Trichuris* eggshells, the homogenate was sonicated while frozen at -20 ˚C using ten cycles of 10 x 1-second pulses at maximum intensity with a Microson Ultrasonic Cell Disruptor XL™ (Misonix). Homogenates were checked for eggshell disruption under a stereomicroscope (10x - 30x magnification), centrifuged (10,000 x $g$ for 10 min at 4 ˚C) and the supernatant containing the soluble NE egg proteins recovered (the *T. trichiura* NE egg extract - EE) and frozen at -20 ˚C until further analysis.
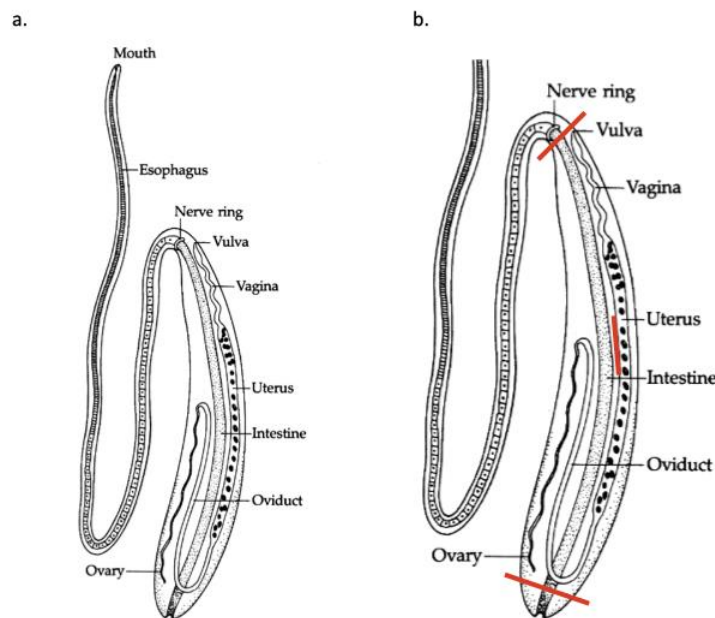


**Figure 3.1 *Trichuris trichiura* uterus dissection guide.**
(A) Identification of anatomical landmarks. (B) identification of the three dissection zones.

### 3.2.4 Protein quantification of parasite extracts

The total protein concentrations of the extracts (AE and EE) were determined using a commercial Protein Assay (Bio-Rad®, CA, USA) according to the manufacturer's instructions with Bovine Serum Albumin (BSA) (Sigma-Aldrich®) as the standard. The assay uses the Bradford method (Bradford, 1976) to quantify soluble proteins (1-10 µg/mL) that is based on the binding of Coomassie Brilliant Blue G-250 dye to form a dye-protein complex which can be detected at 595 nm in a spectrophotometer (ELISA iMark Microplate Absorbance Reader (Bio-Rad®)) using the software Microplate Manager™ and quantitated against the BSA (Sigma-Aldrich®) standard quantification curve generated.

### 3.2.5 Preparation for Scanning Electron Microscopy (SEM)

Specimens were removed from Karnovsky's fixative and washed four times in 0.1 M cacodylate buffer (stock solution with 0.2 M sodium cacodylate to 1 L distilled $H_2O$). Following dehydration in 50%, 70%, 80% and 90% ethanol for 15 min each and a final incubation in 100% ethanol for 1h, the specimens were dried in a Critical Point Dyer (Balzer Union, Balzers, Liechtenstein) and mounted on the carbon mounts sample plates. Gold palladium vaporation was carried out at 25 mA for 8 min. The samples were prepared and analyzed in a scanning electron microscope (Zeiss Auriga, FEI QemScan 650 F, Oberkochen, Germany) at the Center for Scientific Instrumentation (CIC) of the University of Granada.

### 3.3 African green monkeys (AGM; *Chlorocebus sabaeus*)

### 3.3.1 Animals

Apparently healthy juveniles and adult *C. sabaeus* (11 females and 22 males) humanely euthanized for other experiments performed at the SKBRF & Virscio were used for the collection of samples. Prior to euthanasia the animals were maintained and treated according to standard operating procedures in place at the facility for cleaning, feeding and enrichment activities (see section **3.4.1**).

### 3.3.2 Feces and analysis

Feces were collected from individual *C. sabaeus* cages according to SKBRF & Virscio standard operating procedures. They were also collected from *C. sabaeus* following humane euthanasia as described in section **3.2.1**. Aliquots (2 g) were examined within 48 h using the double centrifugation technique with Sheather's sugar flotation solution (spg 1.28) as described previously (Zajac & Conboy, 2012) (**Appendix II**).

### 3.3.3 Sera

Blood was drawn according to SKBRF & Virscio standard operating procedures as part of other studies. Sera were separated by centrifugation at 2,000 x $g$ for 15 min at room temperature and stored at -80 ˚C until analysis.

### 3.3.4 Saliva

Saliva was collected following an in-house protocol (**Appendix III**). In short, when *C. sabaeus* were anaesthetized during the morning for other studies conducted at the SKBRF & Virscio with ketamine HCl 8 mg/kg (Bioniche Pharma USA LLC, IL, USA) and xylazine 1.6 mg/kg (AnaSed® Akorn, Inc., IL, USA). Cotton rolls (Richmond™, NC, USA) were placed along the upper and lower mucobuccal folds and left for 5 min before being placed in 3 mL sterile syringes (BD Luer-Lok™ tip, NJ, USA) for transport on ice to the RUSVM research laboratory. Within 3 h of collection, each syringe with its cotton roll was placed, needle-attachment site down, into a 15 mL centrifuge tube (Falcon™, NY, USA) and centrifuged for 20 min at 1,500 x $g$ at 4 °C. The supernatant saliva was removed from the Falcon™ tube, mixed with equal volumes of saliva conservation mix (PBS, cOmplete™ mini EDTA-free Roche® and ProClin® Sigma-Aldrich) (**Appendix IV**), and stored at -80 ˚C until analysis.

### 3.3.5   Human sera and saliva

Saliva and sera from humans to be used as negative controls were obtained from other studies where the study subjects or volunteers had agreed to their anonymized samples being used for other research. The samples were stored at -80 ˚C until use.

### 3.4   Case report on nematode control of captive AGMs

### 3.4.1   Animals and Facilities

St. Kitts Biomedical Research Foundation (SKBRF) and translational research affiliate Virscio, Inc. are an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)-accredited biomedical research facility for integrated preclinical research and development with a multidisciplinary nonhuman primate translational focus. The facility houses approximately 1,000 *C. sabaeus,* primarily in outdoor social enclosures or indoor pair house caging designed according to regulations in the *Guide for the Care and Use of Laboratory Animals* (National Research Council of the National Academies, 2011).

All the *C. sabaeus* on which we collected data were being used in studies approved by the Institutional Animal Care and Use Committee of SKBRF & Virscio. The AGMs had entered the facility between January 2018 and July 2019 and were housed and cared for according to SKBRF standard operating procedures. All the animals were moved to individual stainless steel cages that meet the nonhuman primate requirements (National Research Council of the National Academies, 2011) for the duration of their study periods, except for AGM 20 that remained in paired housing and AGMs 21-26 with longest study periods of 12 – 24 months that spent a range of 4 to 14 months in social enclosures according to standard regulations (National Research Council of the National Academies, 2011). The individual cages have steel mesh floors lined with steel pans above the ground that meet standard non-human primate requirements (National Research Council of the National Academies, 2011) (minimum height 30 cm$^2$ and floor space 0.4 m$^2$). The cages were washed down with water daily to remove feces, urine, and left-over food debris.

Every two weeks the AGMs were removed from the cages, which were then sanitized using a combination of steam (>82 ˚C) and a quaternary-based disinfectant (Consume Eco-Lyzer®,

Consume Nature's Way, Spartan Chemical Company Inc, Ohio, USA), or using an accelerated hydrogen peroxide foam (Peroxigard ™, Virox® Technologies Inc. Ontario, Canada). The residual disinfectant was removed by power washing with cold water. The disinfectant used was alternated at each 2-week application, and sanitation effectiveness measured by routine Adenosine triphosphate (ATP) bioluminescence testing of randomly selected surfaces after cages had dried after the final power wash.

The main food provided once a day to the *C. sabaeus* was monkey chow (Envigo® Teklad 8773 primate biscuits, Indiana, USA) which was supplemented with seasonal fruits and vegetables sourced from on-site gardens or local farms. The fruits and vegetables were picked directly from the trees and washed if evidently soiled, they were provided for both nutrition and as an enrichment activity for the animals' wellbeing. Water, provided *ad libitum* with Lixit® (California, USA) water valves in the individual cages, was from the St. Kitts main water supply which the facility filtered (Neo-Pure PS-27097-05 9-3/4" standard efficiency pleated filter 5 μ, Neologic solutions©, South Carolina, USA) and treated with ultraviolet light (Sanitron© ultraviolet water purifiers, Altlantic Ultraviolet Corporation®, New York, USA). The water lines in the facility were sanitized monthly with a sodium hypochlorite flush delivered by an Edstrom automated watering system (Avidity Science©, Wisconsin, USA).

### 3.4.2   Treatments

Until recently, based on Association of Primate Veterinarians recommendations (APV) (Nonhuman primate formulary, 2018; Calle *et al*., 2014), wild-caught *C. sabaeus* brought into the facility were routinely treated with intramuscular 5mg/kg praziquantel (Droncit®, Kansas, USA) and 15 mg/kg albendazole (Valbazen®, Michigan, USA) (PRAL treatment) delivered by nasogastric or orogastric gavage to chemically restrained animals (ketamine HCl, 8 mg/kg, Bioiniche Pharma USA LLC and xylazine, 1.6 mg/kg, AnaSed ® Akorn, Inc.) animals. This treatment was repeated after 2 weeks and arbitrarily every 3-5 months for ongoing control.

In 2019, a new treatment regimen was introduced based on a report from Kenya (Kagira *et al.*, 2011) that a combination of ivermectin and albendazole (IVAL treatment) resulted in a 100

% fecal egg reduction rate for *T. trichiura* and strongyles. The IVAL treatment we used consisted of a three-day regimen of 300 μg/kg ivermectin (Noromectin®, Newry, Co. Down, Northern Ireland) SQ SID, and 10 mg/kg albendazole (Valbazen®) SID delivered by nasogastric or orogastric gavage following chemical restraint, as described above. The IVAL treatment was repeated when an animal had a positive fecal flotation result during routine screening after three months. The praziquantel treatment regimen remained used for cases diagnosed with cestode or trematode infections.

### 3.4.3 Monitoring of treatments

We monitored the success of the PRAL and IVAL treatments in *C. sabaeus* that required sampling and necropsy as part of studies in which they were enrolled and that had been approved by the Institutional Animal Care and Use Committee of SKBRF & Virscio. The *C. sabaeus* treated with the PRAL regimen (n= 26; 9 females, 17 males) had FECs (all AGMs) and counts of adult *T. trichiura* in the large intestine (15 AGMs – see below) performed at routine necropsies carried out 2, 4, 5, 10, 12, 15, 22 and 24 months after the initial PRAL treatment and at least 3–5 months after ongoing PRAL treatments in the case of the AGMs euthanized 10 – 24 months after the initial treatment.

The *C. sabaeus* treated with the IVAL regimen (n=11, all males) had FECs carried out on feces collected from their cages 3 months after the IVAL treatment. Animals with a positive result were given a second IVAL treatment and all animals tested by FEC and counts of adult *T. trichiura* in the large intestine at routine necropsies 6 or 8 months after the initial IVAL treatment.

### 3.4.4 Morphological identification

Parasites were identified to the species level whenever possible as detailed species-level taxonomic identification from microscopic analysis alone is not always feasible. Morphologic identification of *T. trichiura* eggs was based on size (50-60 x 20-30 μm), brown-bile green color, the presence of bipolar plugs, and the characteristic lemon-shaped unembryonated egg with a smooth shell, an important characteristic to differentiate from *Capillaria*. Specimens of *Capillaria*

spp. were identified based on size (50-60 x 20-30 µm), ovoid barrel-shaped unembryonated egg, light yellow/green color, bipolar prominences, and characteristic striated shell. Eggs were classified as *Strongyloides* spp. based on size (50-60 x 30-40 µm), lack of color, ellipsoidal shape, thin shells, and the presence of larvae coiled inside the eggs was regarded as an indication of *Strongyloides fuelleborni* (Acha *et al.*, 2003; Modrý *et al.*, 2015). Eggs were classified as strongylid based on their size (60-85 x 35-50 µm), ellipsoidal shape, and their smooth, thin shelled and colorless appearance. Strongylid eggs containing a morula were regarded as hookworms, while those slightly longer, with an early cleavage-stage embryo and one or both eggshell ends more pointed were classified as *Trichostrongylus* spp. (Hasegawa, 2009; Modrý *et al.*, 2015; CDC, 2016) (**Figure** 3.2).
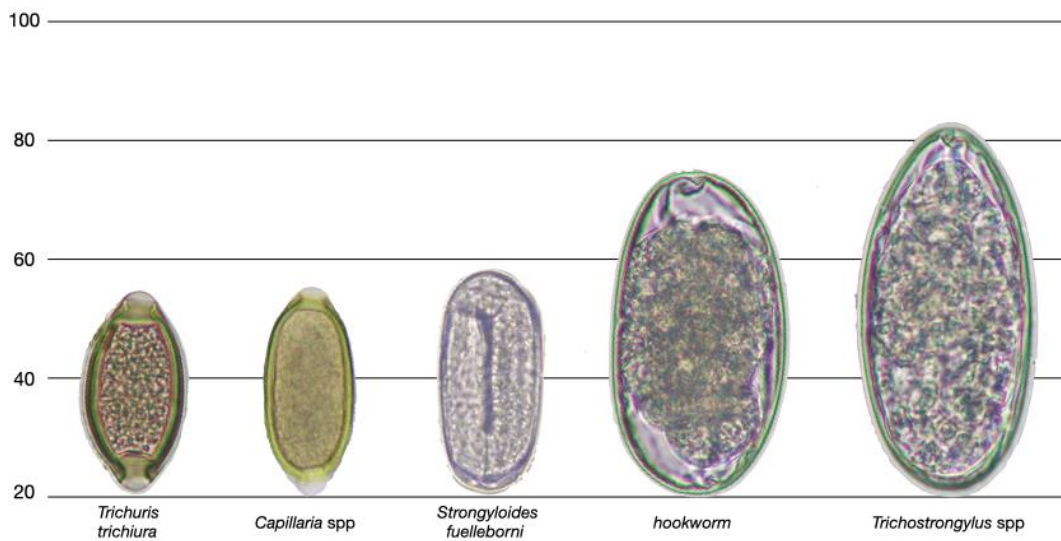


Figure 3.2 Morphologic identification of nematode eggs species found.

Measurements in µm.

## 3.5 Identification of immunogenic proteins of diagnostic value in *T. trichiura* adults and eggs

### 3.5.1 Indirect ELISA for detection of specific antibodies anti-*Trichuris* in sera and saliva of infected animals.

Microtiter plates (96 well; Thermo Fisher Scientific, MA, USA) were coated with 5 µg of AE diluted in coating buffer 0.05 M, pH 9.5-9.6 ($Na_2CO_3$, $NaHCO_3$) in a final volume of 100 µL per well. After overnight incubation at 4 ˚C the coating buffer was removed, and the plates were blocked with BSA (Sigma-Aldrich®) 0.05% in PBS-0.05% Tween 20® (Sigma-Aldrich®) (PBST) for 1h at 37 ˚C on an orbital shaker at 60 rpm. Duplicate samples of saliva (1/80 in PBST) and sera from infected AGMs and negative human controls (1/100 in PBST) were incubated in wells for 90 min at 37 ˚C on an orbital shaker at 60 rpm. After three washes in PBST the plates were incubated for 1h at 37 ˚C on an orbital shaker at 60 rpm and reactive antibodies in AGM samples detected with peroxidase labeled goat anti-primate IgA or IgG (Novusbio™ Novus Biologicals LLC, CO, USA) (1/5,000 in PBST), and for human samples goat anti-human IgA or IgG (Abcam®, Cambridge, UK) (1/5,000 in PBST). After washing as above, immune complexes formed were revealed with OPD substrate (o-Phenylenediamine dihydrochloride) (Sigma-Aldrich®) 0.4% mg/mL in 0.05 M, pH 5 citrate -phosphate buffer (0.1 M $C_6H_8O_7$/0.2M $NaHPO_4$ 0.2 M/30% $H_2O_2$,). After 5 min at room temperature (21˚C), the reaction was stopped with 3N HCl and the colorimetric reaction assessed photometrically at 490 nm using iMark Microplate reader (Bio-Rad®) and Microplate Manager 6.0 (Bio-Rad®).

Each sample was tested in duplicate, and the results expressed as mean optical densities (OD) with their standard deviations (SD). The cutoff was established as the mean OD of the negative controls plus 3 SDs.

### 3.5.2 One dimensional *SDS-PAGE* electrophoresis (1-DE) gel staining and Western-blot with serum and saliva

The AE and EE (10 µg/well) was diluted in 4x Laemmli buffer (Bio-Rad®) (1:1), denatured at 100 ˚C for 5 min under reducing conditions and separated by electrophoresis in Mini-Protean® TGX precast acrylamide gels (4-15% gradient, 10 well comb, 50 µL/well) (Bio-Rad®) with 80 − 120

V in a Mini-PROTEAN Tetra System electrophoresis system (Bio-Rad®). Samples were run simultaneously with molecular weight markers (4 µL) (Precision Plus Protein ™ Dual Color Standards, Bio-Rad®). Following electrophoresis, half of the gel was stained with Coomassie brilliant blue R-250 (Bio-Rad®) and the other mirror half transferred onto nitrocellulose filters (Trans-Blot Turbo™ transfer pack, Mini Format, 0,2 µm Nitrocellulose, single application, cat # 1704158, Bio-Rad®) using a Trans-Blot® Turbo™ transfer system (Bio-Rad®) as per manufacturer's instructions for mixed MW for 7 min. The blotted membrane was stained in acidic Ponceau S (99 mL distilled water, 0.5 g Ponceau S (Sigma-Aldrich®), 1 mL glacial acetic acid) and after the proteins were visualized, the membrane was washed with distilled water, blocked with 5% skimmed milk in PBST for 2 h at room temperature, and incubated overnight at 4˚ C with a pool of either saliva or serum samples diluted 1:10 or 1:500 in PBST, respectively. Following three washes, each of 30 min in PBST, the membranes were incubated for 4 h at room temperature with the secondary antibody (peroxidase-labeled goat anti-primate IgG (1:5000 in PBST) or IgA (1:1000 in PBST), (Novusbio™), washed three times in PBST (30 min each) and the assay developed using Clarity™ Western ECL substrate (Bio-Rad®) mixed in a 1:1 ratio. The positive reactions were determined by the appearance of defined protein bands detected by chemiluminescence with an Amersham™ Imager 600 (GE Healthcare®, IL, USA). The relative molecular masses of the recognized protein fractions were determined by comparison with molecular weight markers (kDa) and data analysis was completed as previously described (Bernal *et al.*, 2006).

### 3.5.3 Two-dimensional SDS-PAGE electrophoresis (2-DE), gel staining and Western-blot with serum

Egg extract (1 µg/µL) was purified with the 2-D Clean-up Kit (GE Healthcare®, New Jersey, USA) following the manufacture's protocol. After the final centrifugation step, the protein pellets were resuspended in lysis buffer (7 M Urea, 2 M thiourea, 4% CHAPS), and quantified (1 µg/µL) with the RC DC™ Protein Assay (Bio-Rad®).

The EE (10 µg for Coomassie staining, and 30 µg for 2-DE Immunoblotting) was diluted 1:1 in sample buffer containing 65 mM dithiothreitol (DTT) and 1% ampholytes, loaded onto previously hydrated (125 µL DeStreak™ and 1 % ampholytes) 7 cm, pH 3-11 strips (Immobile

DryStrips™ GE Healthcare) for first dimension separation. The protein samples were separated according to isoelectric point (pI) values through Isoelectric focusing (IEF), using a Ettan IPGphor 3 (GE Healthcare®) at 20 ˚C as follows: (i) 300 V for 30 min (step and hold), (ii) 1000 V for 30 min (gradient), (iii) 5,000 V for 80 min (gradient) and (iv) 5,000 V until 4,000 Vh (step and hold) (current limit of 70 µA/IPG strip).

After focusing, the strips were equilibrated in two steps in a reswelling tray (Immobiline™ Drystrip Resweling Tray, Pharmacia Biotech AB®, Uppsala, Sweden) at room temperature. The first step comprised 15 min in equilibration buffer (BE) (6 M Urea/ 50 mM Tris/HCL, pH 8.8/ 29.3% Glycerol/ 2% SDS/ 0.002% Bromophenol blue) with 2% DTT (GE healthcare®), and the second step was carried out for 15 min in BE with 2.5% iodoacetamide (IAA) (GE healthcare®). The two-dimensional SDS-PAGE was performed with 12.5 % acrylamide separating gels in a Mini-PROTEAN Tetra Cell electrophoresis chamber (Bio-Rad ®) at 60 – 120 V for 120 min (Bio-Rad®). The samples were run simultaneously with 5 µL of the molecular weight marker (Precision Plus Protein ™ Dual Xtra Standards; Bio-Rad®) in the Coomassie gel and 2 µL in the 2D WB gel.

After 2-DE, resultant EE gels were stained with Coomassie brilliant blue R-250 (Bio-Rad®) or transferred to nitrocellulose paper (Amersham ™ Hybond ™-ECL, GE healthcare®) using the Mini Protean ™ transfer system (Bio-Rad®) at 200 mA for 2 h. The blotted membrane was processed for Western blotting as described for 1-DE above except the incubation period of the secondary antibody was 4 h.

### 3.6 Proteomic analysis of *T. trichiura* AE and EE

Sample preparation, liquid chromatography, tandem mass spectrometry and initial proteomic analysis of MS/MS fragmentation data was performed for the study of *T. trichiura* complete egg proteome and immunogenic bands from egg (EE) and adult (AE) extracts by the proteomics facility of *Servei Central de Suport a la Investigació Experimental* (*SCSIE*) of University of Valencia (Burjassot, Spain) that belongs to ProteoRed, PRB2-ISCIII, supported by grant PT13/001.

### 3.6.1 Sample preparation

Following electrophoresis, the strip of gel containing the all the EE products was cut and digested with 500 ng of sequencing grade trypsin (Promega, WI, USA), a proteolytic enzyme, in 200 µL of ammonium bicarbonate solute. The selected immunogenic bands visible and non-visible (in the case of immunogenic bands that are not clearly visible in the SDS-PAGE due to lower abundance) were identified on the SDS-PAGE Coomassie gels and cut for digestion with 100 ng of sequencing grade trypsin (Promega) in 100 µL of ammonium bicarbonate solution as described elsewhere (Shevchenko *et al.*, 1996). Digestion was stopped with 1% trifluoracetic acid (TFA) and a double extraction with acetonitrile (ACN) performed as previously described (Bernal *et al.*, 2006). The final peptide solutions were vacuum dried in a rotatory evaporator and resuspended with 25 µL of 2% ACN and 0.1% TFA (pH 2) for EE and 9 µL 2% ACN and 0.1% TFA (pH 2) for the individual bands.

### 3.6.2 Liquid chromatography and tandem mass spectrometry (LC-MS/MS)

To initiate the elution process, 5 µL of the final peptide solution was loaded onto a trap column (Nano-LC Column, 3µm C18-CL, 350 µm x 0.5 mm, Eksigent ®, AB SCIEX©, CA, USA) and desalted with 3 µL/min 0.1% TFA over 5 min. The peptides were loaded onto an analytical column (LC Column, 3 µm C18-CL, 75 µm x 12 cm, Nikkyo, Nikkyo Technos Co., Ltd, Tokyo, Japan) equilibrated in 5% acetonitrile, 0.1% formic acid (FA) and eluted using a linear gradient (5-35%) of solvent B (0.1% FA in ACN) in A (0.1% FA) for 120 min for the EE and 30 min for the individual bands at a flow rate of 300 nL/min. The eluted peptides were analyzed with a nanoESI-Q-TOF mass spectrometer (5600 TripleTOF, AB SCIEX©) in an Information Dependent Acquisition Mode (IDA) a per manufacturer's instructions. An IDA method automatically runs experiments based on results obtained from previous experiments using Analyst ®, AB SCIEX© software.

The eluted sample was ionized applying 2.8 kV to the spray emitter and survey MS1 scans were acquired from 350 to 1,250 m/z for 250 ms. The quadruple resolution was set to 'UNIT' for MS2 experiments, which were acquired 100–1,500 m/z for 50 ms in 'high sensitivity' mode. The following switch criterion was used: charge 2+ to 5+, minimum intensity, 70 counts per second

(cps). Up to 50 ions were selected for fragmentation after each survey scan. Dynamic exclusion was set to 15 s. The system sensitivity was controlled with 2 fmol of 6 proteins (LC Packings, A Dionex Company, Amsterdam, Netherlands).

### 3.6.3 Bioinformatic analysis

ProteinPilot™ Software (version 4.5.1, revision 2768; Paragon™ Algorithm 4.5.1.0, 2765; Applied Biosystems®/MDS Sciex, MA, USA) default parameters were used to generate the peak list directly from 5600 TripleTof .wiff files. All .wiff files from the sample were combined in a single search. ProteinPilot™ Software is a program used for analysis of peptide tandem mass spectrometry data by conducting protein inference analysis and relative quantification of proteins. The Paragon™ Algorithm (Shilov, I.V., *et al*., 2007) included in ProteinPilot™ software was used for searching the National Center for Biotechnology Information (NCBI) protein database with the following parameters: tryptic specificity, cys-alkylation, Metazoa, Nematoda and *Trichuris trichiura* protein taxonomy restrictions. The Paragon™ scoring algorithm provides confidence levels for proteins hits.

Protein grouping was done by Pro Group™ algorithm. A protein group in a Pro Group™ Report is a set of proteins that share some physical evidence, meaning that they are groups of related and homologous proteins.

Unlike sequence alignment analyses where full length theoretical sequences are compared, the information of protein groups in Pro Group™ is guided entirely by observed peptides alone. Since the observed peptides are actually determined from experimentally acquired spectra, the grouping can be considered to be guided by usage of spectra. Therefore, unobserved regions of protein sequence play no role in explaining the data.

Identification was considered accurate when the ProteinPilot™ *Unused score* (see definition in section **3.6.4**) greater than 1.3 was identified with ≥ 95% confidence according to the following equation:

ProtScore = - log (1 – (percent confidence/100))

The resulting proteins identified in each sample are presented in the *Proteins Detected Table* (see definition in section **3.6.4**) with the following information for each of them.

### 3.6.4 Proteomic results interpretation glossary

*Proteins Detected Table*

The Proteins Detected Table lists the winner protein for each group, sorted by *Unused ProtScore*.

*N*

The rank of the specified protein relative to all other proteins in the list of detected proteins.

*Unused (ProtScore)*

The Unused ProtScore reflects the amount of total, unique peptide evidence related to a given protein. It is a measure of the protein confidence for a detected protein, calculated from the peptide confidence for peptides from spectra that are not already completely "used" by higher scoring winning proteins.

*Total (ProtScore)*

A measure of the total amount of peptide evidence related to a detected protein. The Total ProtScore is calculated using all of the peptides detected for the protein. The Total ProtScore does not indicate anything about the confidence that a protein has been detected, because some or even all of the spectra contributing to the Total ProtScore may be better explained by higher ranked proteins.

*% Cov (Coverage)*

The percentage of matching amino acids from identified peptides having confidence greater than 0 divided by the total number of amino acids in the sequence.

*% Cov (60)*

The percentage of matching amino acids from identified peptides having confidence greater than or equal to 60% divided by the total number of amino acids in the sequence.

*% Cov (95)*

The percentage of matching amino acids from identified peptides having confidence greater than or equal to 96% divided by the total number of amino acids in the sequence.

*Accession #*

The accession number for the protein corresponding to the peptide sequence transcript ID code.

*Name*

The name of the protein.

*Species*

The species code for this protein. Not all FASTA files have species, so this column may be blank.

*Peptides (95%)*

The number of distinct peptides having at least 95% confidence. Multiple modified and cleaved states of the same underlying peptide sequence are considered distinct peptides because they have different molecular formulas (**Figure** 3.3). Multiple spectra of the same peptide, due to replicate acquisition or different charge states, only count once.

| N | Unused | Total | %Cov | %Cov(50) | %Cov(95) | Accession | Name | Species | Peptides(95%) |
|---|--------|-------|------|----------|----------|-----------|------|---------|---------------|
| 1 | 261.11 | 261.11 | 73.72999787 | 71.79999948 | 65.71000218 | TTRE_0000643201-mRNA-1 | transcript=TTRE_0000643201-mRNA-1 gene=TTRE_0000643201 | 0000643201-mRNA-1 | 470 |
| 2 | 6.04 | 6.04 | 10.88000014 | 9.296999872 | 7.710000128 | TTRE_0000228401-mRNA-1 | transcript=TTRE_0000228401-mRNA-1 gene=TTRE_0000228401 | 0000228401-mRNA-1 | 4 |
| 3 | 5.21 | 5.27 | 14.63000029 | 14.63000029 | 14.63000029 | TTRE_0000521801-mRNA-1 | transcript=TTRE_0000521801-mRNA-1 gene=TTRE_0000521801 | 0000521801-mRNA-1 | 4 |
| 3 | 4.24 | 4.26 | 20.6400007 | 18.60000044 | 11.63000017 | TTRE_0000829301-mRNA-1 | transcript=TTRE_0000829301-mRNA-1 gene=TTRE_0000829301 | 0000829301-mRNA-1 | 3 |
| 4 | 3.05 | 3.13 | 3.946999833 | 3.946999833 | 3.946999833 | TTRE_0000682001-mRNA-1 | transcript=TTRE_0000682001-mRNA-1 gene=TTRE_0000682001 | 0000682001-mRNA-1 | 2 |
| 5 | 2.17 | 2.18 | 40.63000083 | 23.52000028 | 13.16000074 | TTRE_0000464201-mRNA-1 | transcript=TTRE_0000464201-mRNA-1 gene=TTRE_0000464201 | 0000464201-mRNA-1 | 1 |
| 6 | 2 | 2 | 12.5 | 7.500000298 | 7.500000298 | TTRE_0000189701-mRNA-1 | transcript=TTRE_0000189701-mRNA-1 gene=TTRE_0000189701 | 0000189701-mRNA-1 | 1 |
| 7 | 1.68 | 1.72 | 1.095999964 | 1.095999964 | 1.095999964 | TTRE_0000430801-mRNA-1 | transcript=TTRE_0000430801-mRNA-1 gene=TTRE_0000430801 | 0000430801-mRNA-1 | 1 |
| 8 | 1.46 | 1.49 | 4.467000067 | 4.467000067 | 4.467000067 | TTRE_0000107401-mRNA-1 | transcript=TTRE_0000107401-mRNA-1 gene=TTRE_0000107401 | 0000107401-mRNA-1 | 1 |

Figure 3.3 Example of Proteins Detected Table from bioinformatics analysis database.

Because proteins can often share homology (similarity at the sequence level), there are often peptides identified in a database search that point to more than one protein. The Pro Group™ Algorithm works to try to resolve the complexity of reporting identified proteins by selecting only the ones that are truly present. After the peptide identification is complete a Pro Group Algorithm is run to assemble the list of proteins, the *Total ProtScore* of every protein is computed and then ranked. Starting at the top of the list, all the peptide evidence is assigned to the first protein, making the *Unused ProtScore* the same as the *Total ProtScore.* Then each protein down the list is analyzed and when shared peptide evidence is found for a protein that has already been used in a protein higher up the list, the score for that peptide is removed from the lower protein and the *Unused ProtScore* is recalculated. Therefore, the *Unused ProtScore* will be slightly less than the *Total ProtScore* and will reflect only the unique evidence that supports the presence of that protein.

### 3.6.5   Proteomic analysis: Protein identification

Protein identification from the *Proteins Detected table* of results with 95% confidence scores and highest numbers of distinct peptides were considered significant. For the EE complete proteome analysis, all proteins including those with only one distinct peptide were subject to further identification and for the specific immunogenic bands excised, only the top proteins with the highest number of distinct peptides (minimum of two distinct peptides) identified and highest > 95% coverage scores were subsequently analyzed for identification. The process of identification is described in detail below.

### 3.6.5.1   First analysis: for data base construction

The protein identification for the construction of our own data base was conducted by following three main stages:

1. Entering the resulting peptide sequence transcript ID accession codes (e.g., TTRE_0000643201-mRNA1) from the *Proteins Detected Table* into the Wormbase Parasite database (version WBPSS14 (WS271) https://parasite.wormbase.org accessed 2018 - 2019). This database includes 174 genomes including the *T. trichiura* genome (Foth *et al.*, 2014) produced by the Parasite Genomics group at the Wellcome Trust Sanger Institute in collaboration with Richard Grencis (University of Manchester). Assembly version TTRE2.1, Bio                                         Project                                         PRJEB535 (https://parasite.wormbase.org/Trichuris_trichiura_prjeb535/Info/Index/).

2. Recording the assigned UniProt accession code obtained from the Wormbase Parasite database https://parasite.wormbase.org accessed 2018 - 2019) (e.g., A0A077ZE83).

3. Assigning from the UniProt database (https://www.uniprot.org accessed 2018-2019) their respective gene ontology (GO) information. The GO version 2018 and 2019 were used, Gene Ontology and GO Annotations are a branch of the European Bioinformatics Institute (EMBL-EBI), Europe's flagship laboratory for the life sciences accessed via embedded data on the UniProt database for each protein. For each protein, we recorded: protein description, molecular weight, signal peptide (if present), Genomic DNA translation

accession number, molecular function, subcellular location, and biological process when known (**Figure** 3.4).



Figure 3.4 Example of UniProt page accessed per protein for information retrieval during bioinformatic analysis.

https://www.uniprot.org

For the specific immunogenic bands of interest, the molecular weight of our Western Blot images (semi-log plot of the molecular weight markers against the distance of the migration of the proteins from gel well origin) were compared to the molecular weight of the proteins identified in the *Proteins Detected Table* obtained from UniProt (see mass (Da)) on the figure example above (**Figure** 3.4). Based on this initial molecular weight comparison, 15 preliminary protein candidates were targeted for further analysis. In the case of the complete EE proteome description, the data base constructed was used and described in: *Trichuris trichiura* egg extract proteome reveals potential diagnostic targets and immunomodulators (Cruz *et al.*, 2021).

### 3.6.5.2 Second Analysis: homology determination in relation to other nematodes

The 15 candidate proteins selected underwent the following <u>three step analysis</u>:

1. For each protein the FASTA sequence format was retrieved using the link embedded in UniProt database (**Figure 3.5**).

Example:

```
>tr|A0A077ZE83|A0A077ZE83_TRITR Vitellogenin N and VWD and DUF1943 domain
containing protein OS=Trichuris trichiura OX=36087 GN=TTRE_0000643201 PE=4 SV=1

MGLKVVILTLLAFALYAAGRHQQRLKLLRDTVNYQNVQESYFRINQKYKFKYNGQVKIGV
PDHSNQNSMTRFTAEVTLVKRSEEHFIIRVNNIRLGKQLGNSKDQDEMASFEELEPVEIK
QSDLKILELPVEFTYAEGQVQDLVFQQDDEEWSENLKRGIINLFQIKLQSTDRTSMEEEQ
SALNRIDTESKTNVGTAYRTREKTVEGECDTMYTVSAIDDDDDDNSERGSRMLVTKAIDM
KSCQRRPEVWWNFQFISPCPRCHQLPRSGERSVESSTTIRYQIRGKRDKFLIERVELSND
HVIAQQNADESAVVVKIKASLKLVSSQESNDGSSEMSNFPATGQRVSDLIYSTRDDEHFD
RFYAEGDQHYNDRLFSRRQKGQDKSAALAEIIMKMMRHMKDTADEKANRYFYKAVQLMRY
MSESEIRSTNEHHFGRQQSGMLTPEERERARNIMPNLLAQAGTLSSFRQLADKIANGEIN
PLKAAIVITTMMDTPRVSKEIITELMRLDESQTVQRNEQLRRAILLTAGSMMRTMCAPQR
HQRQQRQESQNRDDIRTDNSNHQRCDSEIKQRFVRTIADRLAASDRWEDQVILIRALGNA
GLDVSISELESIIRNQDRRNPAAIRLEAILALRHIKDSLPQKTKNILLSAAANRMESSAV
RMAAIQMLLQQNPDRMTIDQIGVIINHDPNRRVASFAYKLIRRLADSNQPCYEENKQKLQ
TVAKSVRRRNLQLPHSDMIFESVYDREKKTGFDFFIMPMYDMDDIVPKFMRAGINMVERG
ERNRNLFALEIGTSSLSNIISELLPDIEQNQRGGNTEIKMKLRRMAEETRRNGNRGSRQQ
QQQRQQQQRQTKAWMSMKFRNQDIMLLTFNEDRLKQLTQQNRGAESLLLLIAKMASNRQG
SISIDEATLLRETVVKIPTAIGSQLSIRRKAPAIFSAHGQASIGRNIPIQADIRARISTT
ISMVTDVSSRTPISTNGIYLIKNIKATVPVDMTISLDHRQEDEQLKIQMRNHEGYKDLLK
LESRPVIYYMRSQNPLAEAEEKTVVAEKNVRMESFKRCMRGPLFGSEICMRGVITTPMCN
HNKLLHYMAPWFGPNKVTISAYMRRTEENENENENSRQVGLTIRSDSMQNKLELEYETSS
RSLIPTKVDAKLQRQGHDGQRQEICINMKTQGKENDQRSEQTRRSSDININWGTQCNDEN
YIKARIETASRRNVIWEQRQSNAIQEDNEERDQKQIGYGVSSEETAGYKIQIQHRNVPEW
AVDKAEDIIRMLTSMNYWSTEIENKRQRYDSAERNRQRDEQSRAGEVRIQAIMRNEDKAD
VKIQTPRKTIRMNNVYIPTLLRKETYRRSNFMRLMNLYMGNKHAKGTCQIRQNSITTFDG
AIYRIPFSSCYTILARDSEEDPKFAIMARRSREQPDKKVVKLMTSDHEIELIPERGGGIE
VKVDGQRWDDQQSKHHRAMRIRKRENEVTVDLKRPNVDVHYDGNEITIRVSDKYHGRQTG
MCGNLNADSSDEFSKSNQKNRDDIWETFNEMTIRTDDCQHPQRQEQDDSQQFDSYDSDSD
SNSYDSWEMARQHRSDDSISYDDEEESSMFDSDQQQQQRRDQERNQHYRSLAIDDVLTET
APVRRNKVIDSRGRKCISMRPIESCPEHGYTARGEKEEKEVPYTCLKANDELYHKIGRMQ RQG
TPMDLSNSEMSFTRKETVPRKCISIV
```

Figure 3.5 FASTA sequence format example.

2. Subsequently, each FASTA sequence format was analyzed by protein Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and the results from each of the 15 selected preliminary protein candidates were recorded. Proteins with E-value of 0 and 100% homology to *T. trichiura* and no other nematodes were flagged for further analysis. Proteins showing > 55% of homology with other nematodes were excluded. An exception was made for *Trichuris suis* given the high percentage of homology between *T. trichiura* and *T. suis* genomes (Liu *et al.*, 2012). Although a few cases of temporary patent

*T. suis* infection have been observed in man (Nejsum *et al.*, 2012) the potential natural cross-transmission is not clear and it has not been studied in *C. sabaeus* (**Figure 3.6**).

| | Description | Max score | Total score | Query cover | E value | Ident | Access |
|---|---|---|---|---|---|---|---|
| | Vitellogenin N and VWD and DUF1943 domain contain ing protein [Trichuris trichiura] | 3559 | 3559 | 100% | 0.0 | 100% | CDW58129 |
| | von Willebrand factor type D domain protein [Trichuris suis] | 3055 | 3055 | 100% | 0.0 | 87% | KHJ42225. |
| | hypothetical protein M513_05347 [Trichuris suis] | 3033 | 3033 | 100% | 0.0 | 85% | KFD53841. |
| | hypothetical protein M514_05347 [Trichuris suis] | 3025 | 3025 | 100% | 0.0 | 84% | KFD71631. |
| | Vitellogenin-5 [Caenorhabditis elegans] | 351 | 351 | 97% | 5e-95 | 22% | NP_508589 |
| | Vitellogenin-4 [Caenorhabditis elegans] | 350 | 350 | 97% | 1e-94 | 22% | NP_508612 |
| | Vitellogenin-3 [Caenorhabditis elegans] | 344 | 344 | 97% | 8e-93 | 22% | NP_001294 |
| | put. vitellogenin [Caenorhabditis elegans] | 337 | 337 | 97% | 2e-90 | 22% | CAA26849. |
| | Vitellogenin-6 [Toxocara canis] | 335 | 464 | 82% | 1e-89 | 25% | KHN77828. |
| | hypothetical protein PRIPAC_54065 [Pristionchus pacificus] | 327 | 420 | 81% | 6e-87 | 25% | PDM60240 |
| | hypothetical protein PRIPAC_43917 [Pristionchus pacificus] | 325 | 445 | 81% | 1e-86 | 25% | PDM70712 |
| | hypothetical protein PRIPAC_45330 [Pristionchus pacificus] | 324 | 423 | 77% | 5e-86 | 25% | PDM66105 |
| | vit-6 [Pristionchus pacificus] | 323 | 432 | 81% | 1e-85 | 25% | PDM70654 |
| | hypothetical protein WR25_19855 [Diploscapter pachys] | 322 | 421 | 79% | 2e-85 | 24% | PAV85180. |
| | hypothetical protein PRIPAC_45281 [Pristionchus pacificus] | 318 | 368 | 71% | 2e-84 | 24% | PDM66056 |
| | lipoprotein amino terminal region [Oesophagostomum dentatum] | 311 | 311 | 59% | 5e-84 | 25% | KHJ98709. |
| | hypothetical protein PRIPAC_53880 [Pristionchus pacificus] | 317 | 420 | 81% | 8e-84 | 24% | PDM63523 |
| | RecName: Full=Vitellogenin-6; Short=OTI-VIT-6; Contains: RecName: Full=VT3; Contains: RecName: Full=VT2; Flags: Precursor | 310 | 408 | 78% | 6e-82 | 24% | Q94637.2 |

Figure 3.6 Example of protein (VNVD) FASTA sequence format analysis by protein BLAST.

3.  For each of the 15 preliminary protein candidates we also recorded the GenBank data from the NCBI https://blast.ncbi.nlm.nih.gov accessed 2018-2019 (**Figure 3.7**).

Vitellogenin N and VWD and DUF1943 domain containing protein [Trichuris trichiura]
GenBank: CDW58129.1
Identical Proteins FASTA Graphics
Go to:
LOCUS CDW58129  1709 aa linear INV 23-JUL-2014
DEFINITION Vitellogenin N and VWD and DUF1943 domain containing protein [Trichuris trichiura].
ACCESSION CDW58129
VERSION CDW58129.1
DBLINK BioProject: PRJEB535
DBSOURCE embl accession HG806270.1
KEYWORDS.
SOURCE Trichuris trichiura (human whipworm)
ORGANISM Trichuris trichiura
Eukaryota; Metazoa; Ecdysozoa; Nematoda; Enoplea; Dorylaimia; Trichinellida; Trichuridae; Trichuris.
REFERENCE 1
AUTHORS Foth,B.J., Tsai,I.J., Reid,A.J., Bancroft,A.J., Nichol,S.,Tracey,A., Holroyd,N., Cotton,J.A., Stanley,E.J.,
Zarowiecki,M., Liu,J.Z., Huckvale,T., Cooper,P.J., Grencis,R.K. and Berriman,M.
    TITLE The whipworm genome and dual-species transcriptomics of an intimate host-pathogen interaction
    JOURNAL Nature Genetics
    REFERENCE 2 (residues 1 to 1709)
    AUTHORS Aslett,M.
    TITLE Direct Submission
    JOURNAL Submitted (23-JAN-2014) Pathogen Sequencing Unit, Wellcome Trust, Sanger Institute,
            Wellcome Trust Genome Campus, Hinxton,CambridgeCambridgeshire. CB10 1SA,
            UNITED KINGDOM

Figure 3.7 Example of the GenBank data from the National Center for Biotechnology Information

database (NCBI).

https://blast.ncbi.nlm.nih.gov

### 3.6.5.3   Third analysis: Study of immunogenic potential of each protein and its peptides.

Two step analyses for each of the 15 preliminary protein candidates:

1. We assessed the immunogenic potential of each protein FASTA sequence with the purpose of determining which parts of the protein are more immunogenic and from those peptide sequences which specific peptide sections can act as linear epitopes for B lymphocytes. This analysis could mean that the specific sections selected could activate B lymphocytes to produce antibodies and therefore highlight the potential use for antibody diagnostics. For this analysis we used a B Cell epitope prediction database https://www.iedb.org accessed 2018 and 2019 and selected only the peptide sequences with more than 25 amino acids (**Figure** 3.8).

**Vitellogenin N and VWD and DUF1943 domain containing protein [*Trichuris trichiura*]**

| No. | Start | End | Peptide | Length |
|-----|-------|-----|---------|--------|
| 43 | 1340 | 1365 | LLRKETYRRSNFMRLMNLYMGNKHAK | 26 |
| 1 | 21 | 47 | HQQRLKLLRDTVNYQNVQESYFRINQK | 27 |
| 17 | 539 | 567 | QRHQRQQRQESQNRDDIRTDNSNHQRCDS | 29 |
| 32 | 1031 | 1062 | RSQNPLAEAEEKTVVAEKNVRMESFKRCMRGP | 32 |
| 3 | 94 | 127 | RLGKQLGNSKDQDEMASFEELEPVEIKQSDLKIL | 34 |
| 6 | 167 | 200 | KLQSTDRTSMEEEQSALNRIDTESKTNVGTAYRT | 34 |
| 51 | 1668 | 1701 | ANDELYHKIGRMQRQGTPMDLSNSEMSFTRKETV | 34 |
| 38 | 1211 | 1245 | RRNVIWEQRQSNAIQEDNEERDQKQIGYGVSSEET | 35 |
| 8 | 239 | 276 | DMKSCQRRPEVVWWNFQFISPCPRCHQLPRSGERSVESS | 38 |
| 34 | 1104 | 1146 | RRTEENENENENSRQVGLTIRSDSMQNKLELEYETSSRSLIPT | 43 |
| 27 | 806 | 851 | DIEQNQRGGNTEIKMKLRRMAEETRRNGNRGSRQQQQQRQQQQRQT | 46 |
| 10 | 325 | 385 | SSQESNDGSSEMSNFPATGQRVSDLIYSTRDDEHFDRFYAEGDQHYNDRLFSRRQKGQDKS | 61 |
| 50 | 1535 | 1660 | TDDCQHPQRQEQDDSQQFDSYDSDSDSNSYDSWEMARQHRSDDSISYDDEEESSMFDSDQQQQQQRRDQERNQHYRSLAIDDVLTETAPVRRNKVIDSRGRKCISMRPIESCPEHGYTARGEKEEKE | 126 |

Figure 3.8 Example of predicted B cell epitope results from the B cell epitope prediction data base.

https://www.iedb.org

2. We did a secondary BLAST analysis to each B cell epitope predicted peptide sequences selected from each of the 15 potential candidate proteins. We recorded the E-value and the percentage of homology with *T. trichiura* and with other nematodes for each peptide sequence. Peptides sequences with E-value < 10e and 100% homology with *T. trichiura* and no other nematode except for *T. suis* as explained in the second analysis, were included for further analysis (**Figure** 3.9).

| No. | Start | End | Peptide | Length |
|-----|-------|-----|---------|--------|
| 50 | 1535 | 1660 | TDDCQHPQRQEQDDSQQFDSYDSDSDSNSYDSWEMARQHRSDDSISYDDEEESSMFDSDQQ QQQRRDQERNQHYRSLAIDDVLTETAPVRRNKVIDSRGRKCISMRPIESCPEHGYTARGEKEEK | 126 |
| | | | | |

Figure 3.9 Example of secondary BLAST analysis of 1 out of 13 peptide sequences identified for the VNVD protein.

### 3.6.5.4 Fourth analysis: Study of immunogenic potential of each selected peptide

1. For each selected peptide from each of the 15 potential candidate proteins.

   We analyzed their individual antigenic sites using http://www.bioinformatics.nl/cgi-bin/emboss/antigenic accessed 2018 -2019. Resulting Peptide sections with 15 to 20

amino acids in length and 1.1 antigenicity score were included for further analysis (**Figure 3.10**).



```
OUTPUT FILE  outfile

####################################
# Program: antigenic
# Rundate: Thu  2 Apr 2020 23:07:16
# Commandline: antigenic
#     -auto
#     -sequence /var/lib/emboss-explorer/output/613540/.sequence
#     -minlen 6
#     -outfile outfile
#     -rformat2 motif
# Report_format: motif
# Report_file: outfile
####################################

#===================================
#
# Sequence:       from: 1   to: 125
# HitCount: 4
#===================================

Max_score_pos at "*"

(1) Score 1.119 length 19 at residues 74->92
                       *
 Sequence: YRSLAIDDVLTETAPVRRN
           |                 |
           74                92
 Max_score_pos: 80

(2) Score 1.071 length 6 at residues 4->9
             *
 Sequence: CQHPQR
           |    |
           4    9
 Max_score_pos: 7

(3) Score 1.070 length 7 at residues 100->106
              *
 Sequence: RKCISMR
           |     |
          100    106
 Max_score_pos: 105

(4) Score 1.068 length 8 at residues 108->115
              *
 Sequence: IESCPEHG
           |      |
          108     115
 Max_score_pos: 113


#-------------------------------------
#-------------------------------------
```

Figure 3.10 Example of the antigenic analysis results obtained from the selected peptide sequences.

2. The hydrophobicity of each selected peptide section was analyzed using the Parker Hydrophilicity Prediction tool http://tools.iedb.org/bcell/ (Parker *et al.*, 1986), the cutoff was stablished at 1.4 (**Figure 3.11**).
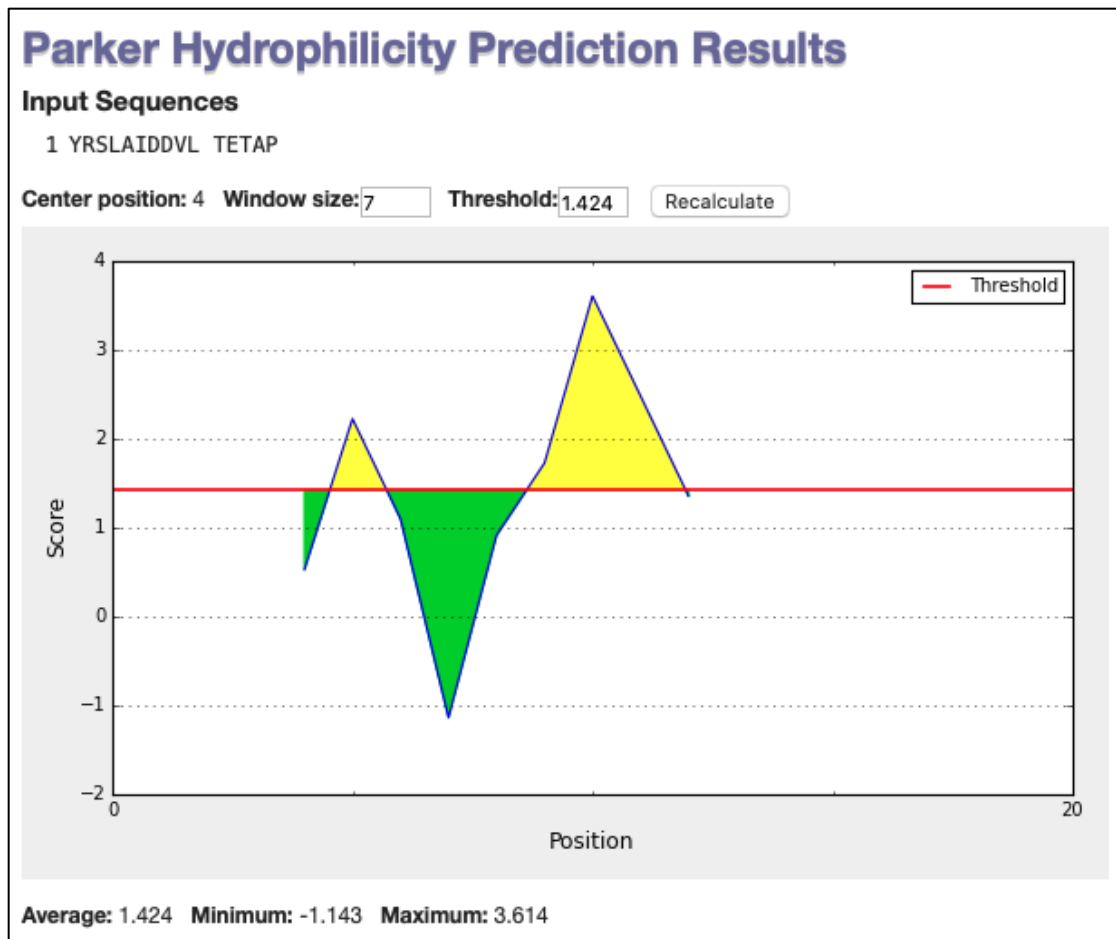
Figure 3.11 Example of Parker Hydrophilicity prediction result for the peptide YRSLADDVLTETAP.

3.  The antigenicity score of each selected peptide section was confirmed by using the Kolaskar and Tongaonkar antigenicity scale prediction tool http://tools.iedb.org/bcell/ (Kolaskar and Tongaonkar, 1990) (**Figure 3.12**).

**Kolaskar & Tongaonkar Antigenicity Results**

**Input Sequences**

1 YRSLAIDDVL TETAP

Center position: 4   Window size: 7   Threshold: 1.000   [Recalculate]

Average: 1.053   Minimum: 1.005   Maximum: 1.119
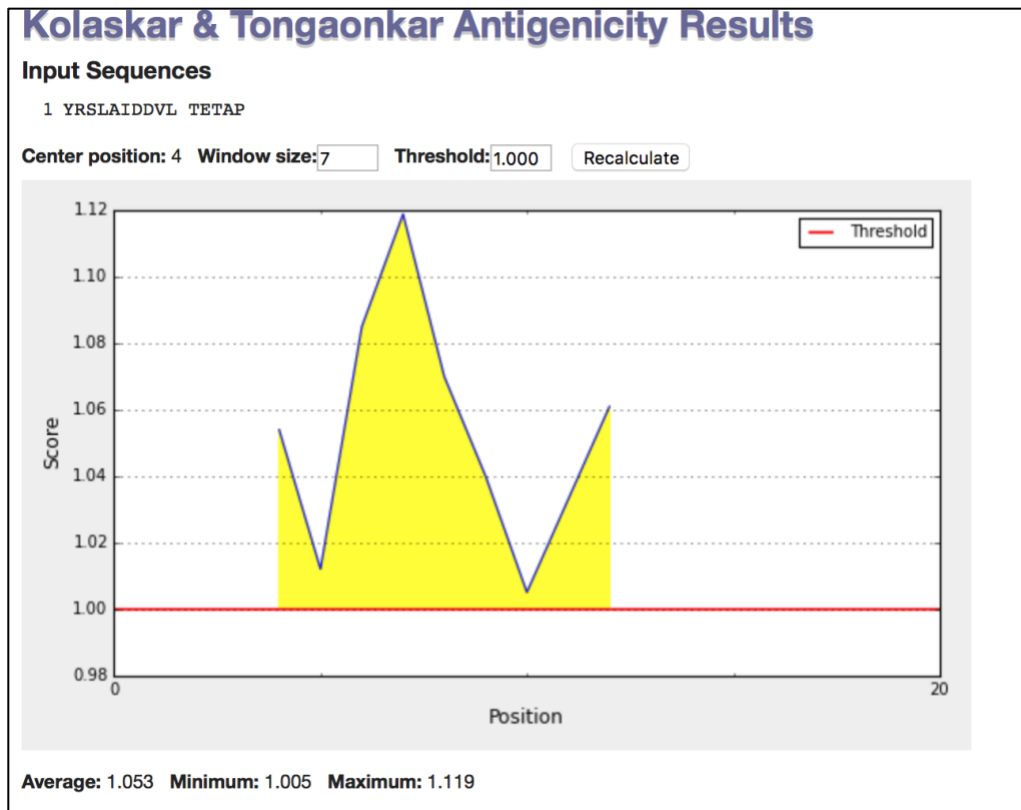
Figure 3.12 Example of Kolaskar & Tongaonkar antigenicity scale prediction tool result for the peptide YRSLADDVLTETAP.

### 3.6.5.5 Fifth analysis: Final BLAST analysis of the individual peptide sections selected from the 15 preliminary protein candidates.

A final step included a BLAST analysis on the final peptide section candidates selected from all previous steps to confirm the specificity of their homology to *T. trichiura* and confirm that the cutoff values stablished initially were still valid (**Figure** 3.13).
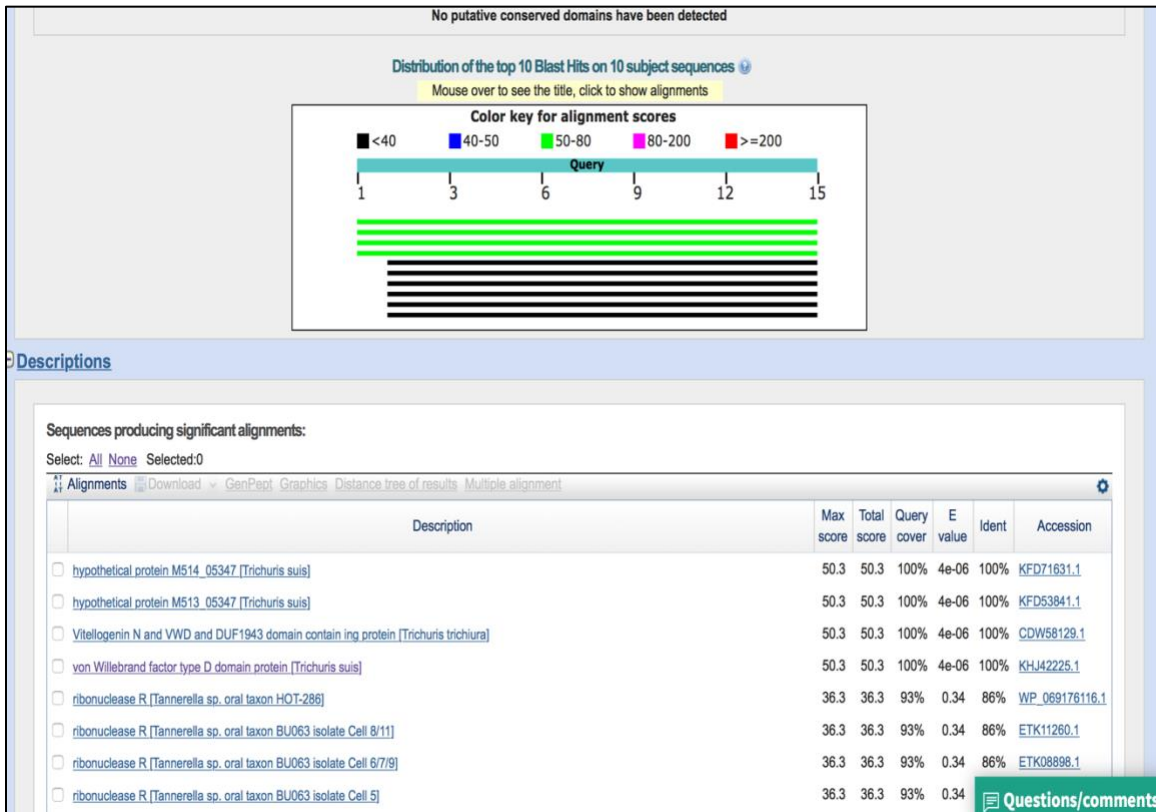
Figure 3.13 Example of final blast analysis for the peptide YRSLAIDDVLTETAP.

The peptide sections that meet the above five bioinformatic comprehensive analysis were selected as final peptide candidates.

## 3.7   Design and evaluation of *T. trichiura* derived synthetic peptides for diagnosis

### 3.7.1   Selection and design of final peptide candidates for ELISA

A total of four synthetic peptides were designed based on the amino acid sequences deducted from the candidate proteins present in the AE and EE, and that were potentially recognized by saliva and serum antibodies from *C. sabaeus* naturally infected with *T. trichiura*.

Peptides were synthetized by CellTein LLC DBA LifeTein LLC, Somerset, NJ (https://www.lifetein.com). The manufacturer quantified the purity and identity of the peptides using High-Performance Liquid Chromatography (HPLC) confirmed by mass spectrometry. The peptides were supplied as a lyophilized product. Each peptide was dissolved in a suitable solvent

(following the manufacturer's instructions) (**Table** 3.2) at a final concentration of 2.25 mg/mL and stored in 200 µL aliquots at − 80 ˚ C.

### 3.7.2    Synthetic peptides

The final candidate proteins from which the peptides were designed were: Vitellogenin N and VWD and DUF1943 domain containing protein, CBM 14 domain containing protein, Kunitz BPTI domain containing protein, Poly-cysteine and histidine tailed protein isoform 2.

Table 3.2 Final candidate proteins and respective peptide selection.

| Peptide | Solvent | Protein | Amino acid sequence | amino acid length |
|---|---|---|---|---|
| 1 | PBS | Vitellogenin N and VWD and DUF1943 | YRSLAIDDVLTETAP | 15 |
| 2 | PBS | CBM 14 | SAPTTVTTQLSPVDCVTL | 18 |
| 3 | Distilled water | Kunitz BPTI | QERCVATLPASVICRLP | 17 |
| 4 | Isopropanol and distilled water. | Poly-cysteine and histidine tailed protein isoform 2 | TECVKPPAHDCPAFG | 15 |

### 3.7.3    Indirect ELISA for testing the applicability of designed peptides

Microtiter plates (96 well; MAXISORP, NUNC-IMMUNO plates, Thermo Fisher Scientific) were coated with 10 µg of each designed synthetic peptide diluted in 0.05 M $Na_2CO_3$, NaHCO3, pH 9.5-9.6 coating buffer in a final volume of 100 µL per well to evaluate the applicability of the in-house ELISA as described in section **3.5.1**.

# 4   RESULTS

## 4.1   Efficacy of treatment analysis and egg to worm correlation in captive *C. sabaeus*

Although AGMs are found widely in Africa there is little data on their internal parasites in their natural habitats (Blersch *et al.*, 2019) and also in captivity in other regions: Barbados (Mutani *et al.*, 2003), Korea (Lee *et al.*, 2010), China (Li *et al.*, 2015) and Brazil (Barbosa *et al.*, 2020). We found a high prevalence of *T. trichiura* (15/26; 56%), strongylids (13/26; 50%) and *Strongyloides* spp. (3/26; 12%) (**Figure** 4.1 and

Table 4.1), which is similar to the situations reported elsewhere.
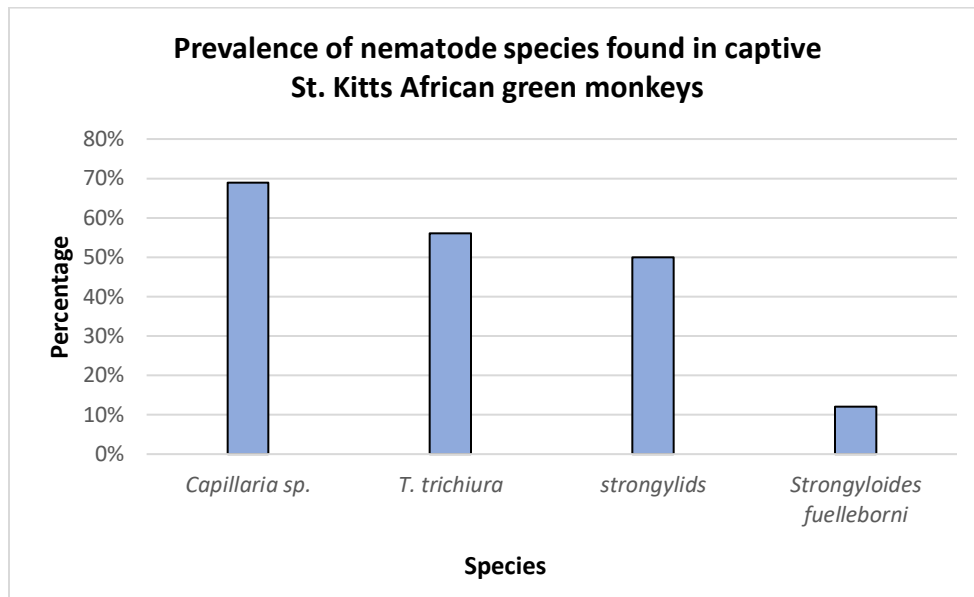


Figure 4.1 Prevalence of nematode species found in captive St. Kitts African green monkeys.

Our finding that *Capillaria* sp. were the most prevalent (18/26; 69%) is of note as the eggs of this genus have not previously been reported in feces of AGMs.

Table 4.1 Outcome of the praziquantel and albendazole treatment regimen (PRAL) on fecal eggs identified 2 to 24 months later and visual identification of adult *Trichuris trichiura* in the large intestine at necropsy.

| Animal number | Months post initial PRAL treatment | *Capillaria* sp. | *T. trichiura* | strongylids | *S. fuelleborni* | *T. trichiura* observed in the large intestine at necropsy |
|---|---|---|---|---|---|---|
| 1 | 2 | - | + | + | - | *NA* |
| 2 | 2 | + | + | + | + | *NA* |
| 3 | 2 | + | - | - | - | *NA* |
| 4 | 2 | - | + | + | - | *NA* |
| 5 | 2 | + | + | - | - | *NA* |
| 6 | 2 | + | + | + | - | *NA* |
| 7 | 2 | + | - | + | - | *NA* |
| 8 | 2 | - | - | - | - | *NA* |
| 9 | 2 | + | + | + | - | *NA* |
| 10 | 2 | + | + | + | - | *NA* |
| 11 | 2 | + | - | - | - | *NA* |
| 12 | 2 | - | - | - | - | - |
| 13 | 4 | + | - | - | - | + |
| 14 | 4 | - | + | - | + | + |
| 15 | 4 | + | + | + | - | + |
| 16 | 4 | + | + | - | - | + |
| 17 | 4 | - | - | + | - | - |
| 18 | 5 | + | + | + | - | + |
| 19 | 5 | - | - | - | - | - |
| 20 | 10 | - | + | - | - | + |
| 21 | 12 | + | - | - | + | - |
| 22 | 15 | + | - | + | - | - |
| 23 | 15 | + | + | - | - | + |
| 24 | 15 | + | + | + | - | + |
| 25 | 22 | + | - | - | - | - |
| 26 | 24 | + | + | + | - | + |
| Totals | | 18/26 | 15/26 | 13/26 | 3/26 | |
| Prevalence | | 69% | 56% | 50% | 12% | |

The efficacy of the PRAL treatment was limited. Only two (2/12; 17%) monkeys that received the traditional PRAL treatment were negative for nematode eggs in FECs performed two months later (**Table** 4.1). Four to five months post-treatment, only 1 (14%) of the seven animals studied was free of nematode eggs, while none (0/7) of the monkeys studied 10 to 24 months post initial treatment were free of eggs despite PRAL dosing for ongoing control every 3 - 5 months and prior to necropsy (**Table** 4.1). No difference was observed in animals that spent time in social enclosures. While *T. trichiura* were not found in the large intestines of the one animal necropsied at 2 months post-treatment, nine of the 14 (64%) monkeys necropsied at 4 to 24 months post initial treatment were infected, four of these as soon as 4 months post-treatment (**Table** 4.1). It is of note that of the nine monkeys that had adult *T. trichiura* in the large intestine, one was negative for eggs by FEC (animal 13). This appeared to be due to a single-sex infection as only two female *T. trichiura* were found in the large intestine of this monkey.



**EPG to total number of adults *T. trichiura***

$y = 0.0227x + 0.8666$
$R^2 = 0.1287$
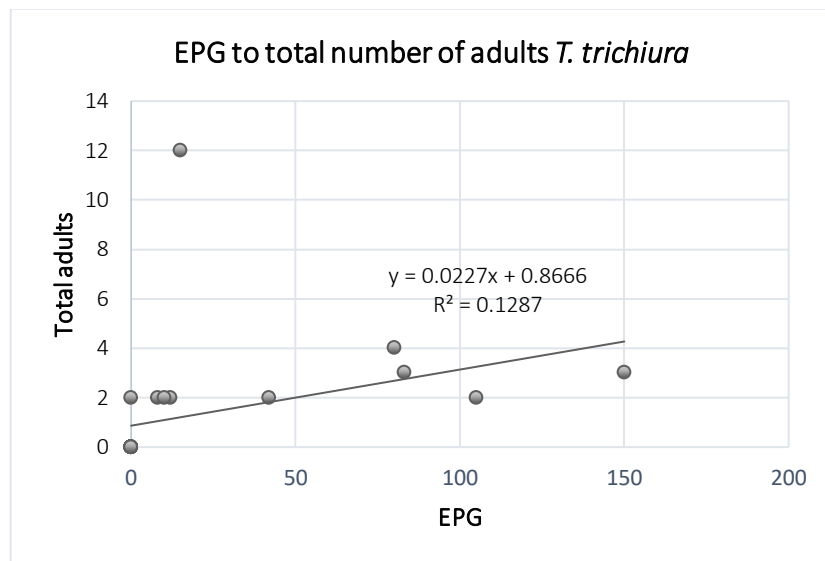
Figure 4.2 Lack of correlation between eggs per gram of feces (EPG) and total adult *T. trichiura* counts from the 26 *C. sabaeus.*

There was no correlation observed when *T. trichiura* FEC were compared with total numbers of adults ($R^2 = 0.1287$) (**Figure** 4.2) or numbers of adult females ($R^2=0.354$) (**Figure** 4.3). This finding provides further evidence that FECs can vary considerably and that an absence of eggs in feces is not reliable evidence of an absence of infection.
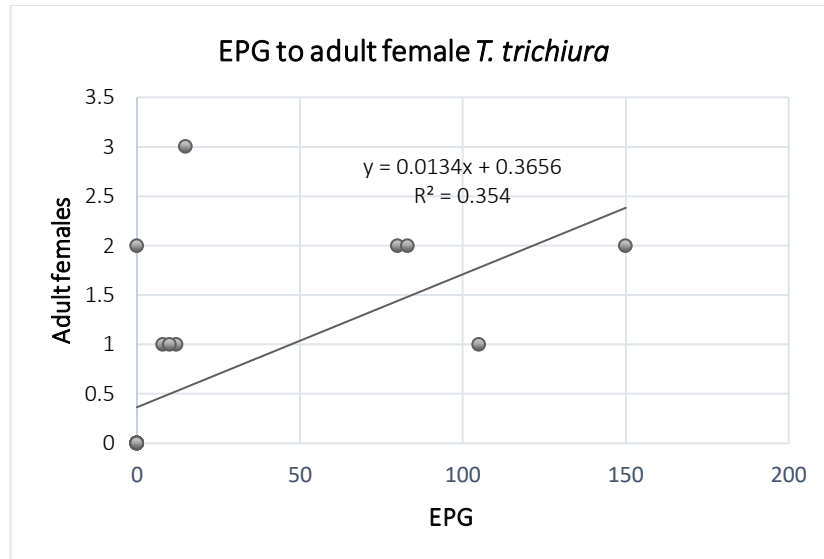
Figure 4.3 Lack of correlation between eggs per gram of feces (EPG) and total female *T. trichiura* counts from the 26 *C. sabaeus.*

Overall, the IVAL treatment regimen together with the husbandry measures in place in our study were relatively successful at controlling nematode infections in captive AGMs. Following a single IVAL treatment, 60% (6/10) of the infected AGMs were negative for nematodes 6 to 8 months later as confirmed by FEC and at necropsy (**Table** 4.2 A). A second IVAL treatment given to the 4 AGMs with persistent *T. trichiura* infections 3 months after the first treatment, resulted in the elimination of the *T. trichiura,* 3 to 5 months later as evidenced by negative FECs and necropsy findings (**Table** 4.2 B). Although apparently cleared of *Capillaria* sp. following the first IVAL treatment, three of these four AGMs had *Capillaria* sp. eggs in their feces at necropsy 3 to 5 months after a second treatment.

Table 4.2 Fecal egg count (FEC) results and adult *Trichuris trichiura* found at necropsy in animals treated with ivermectin and albendazole (IVAL).

A. Animals negative by FEC 3 months after treatment with IVAL were not treated again.

| Animal number | Pretreatment FEC | | | | FEC 3 months after one IVAL treatment | | | | FEC 6 to 8 months after one IVAL treatment | | | | *T. trichiura* at necropsy 6 to 8 months after one IVAL |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *Capillaria* | *T. trichiura* | strongylids | *S. fuelleborni* | *Capillaria* | *T. trichiura* | strongylids | *S. fuelleborni* | *Capillaria* | *T. trichiura* | strongylids | *S. fuelleborni* | |
| 1 | - | + | + | - | - | - | - | - | - | - | - | - | - |
| 4 | - | + | + | - | - | - | - | - | - | - | - | - | - |
| 6 | + | + | + | - | - | - | - | - | - | - | - | - | - |
| 7 | + | - | + | - | - | - | - | - | - | + | - | - | + |
| 8 | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 10 | + | + | + | - | - | - | - | - | - | - | - | - | - |
| 11 | + | - | - | - | - | - | - | - | - | - | - | - | - |

B. Animals positive by FEC 3 months after treatment with IVAL were treated again with IVAL

| Animal number | Pretreatment FEC | | | | FEC 3 months after first IVAL treatment | | | | FEC 3 months after second IVAL treatment | | | | *T. trichiura* at necropsy 3 months after second IVAL |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *Capillaria* | *T. trichiura* | strongylids | *S. fuelleborni* | *Capillaria* | *T. trichiura* | strongylids | *S. fuelleborni* | *Capillaria* | *T. trichiura* | strongylids | *S. fuelleborni* | |
| 2 | + | + | + | + | - | + | - | - | + | - | - | - | - |
| 3 | + | - | - | - | - | + | - | - | - | - | - | - | - |
| 5 | + | + | - | - | - | + | - | - | + | - | - | - | - |
| 9 | + | + | + | - | - | + | - | - | + | - | - | - | - |
| TOTAL | 8/11 (73%) | 7/11 (64%) | 7/11 (64%) | 1/11 (9%) | | | | | | | | | |

## 4.2 *Trichuris trichiura* egg extract proteome reveals potential diagnostic target and immunomodulators

Our results are the first report of the proteome of soluble egg extracts of *T. trichiura* from AGMs (*C. sabaeus*) and describe the potential immunomodulators and antigens recognized by sera and saliva of naturally infected animals.

### 4.2.1 Proteomic characterization of the *T. trichiura* egg extract (EE)

With the spectrometric data obtained using ProteinPilot™ software v4.5 we identified 246 proteins. The unique peptide sequence transcript identification code obtained from the spectrometric data and their respective accession number from WormBase (https://parasite.wormbase.org) enabled us to characterize 231 of the 246 proteins found: 212 had significant homologies with known *T. trichiura* adult stage proteins and 19 were novel uncharacterized proteins with unknown ontology.

### 4.2.2 Gene ontology (GO)

When the proteins were categorized according to their molecular function described in the Gene Ontology (GO) database (UNIPROT; https://www.uniprot.org), 168 were found to have known functions. The different functional groups and biological processes of the most representative proteins of our analysis (with 10 or more distinct peptides) are shown in **Table** 4.3 and **Figure** 4.4. Only a single annotation was assigned to a given protein. Functional annotation of the identified proteins was assigned using GO, which revealed functionally diverse molecules of the common protein families or groups: energy and metabolism; cytoskeleton, motility, and muscle; proteolysis; signaling; stress and detoxification; transcription and translation; and lipid binding and transport (**Table** 4.3). Their specific molecular functions range from molecules involved in ATP, actin, carbohydrate, chitin, lipid, and magnesium ion binding, as well as molecules that take part in oxidoreductase, aminopeptidase, glycogen phosphorylase and metallopeptidase activity (**Table** 4.3). Others include lipid transporter, motor, and protein disulfide isomerase

activity, together with structural constituents of the ribosome or proteins associated with the elongation phase of protein synthesis. Proteins with kinase and intracellular cholesterol transport functions were also identified (**Table** 4.3).

**Table 4.3 Main proteins identified in the EE proteome (10 or more distinct peptides) organized by functional annotation.**

Only a single annotation was assigned to a given protein.

| Functional annotation | Molecular function** | Acc. No. Wormbase | % Cov. | Peptides* (95%) | MW (kDa) | Signal peptide | Biological process** |
|---|---|---|---|---|---|---|---|
| **Energy and metabolism** | | | | | | | |
| Alpha-1,4 glucan phosphorylase | Glycogen phosphorylase activity | A0A077YWK8 | 20.29 | 14 | 101.447 | - | carbohydrate metabolic process |
| ECH domain containing protein | Catalytic activity | A0A077Z1N9 | 44.83 | 17 | 31.202 | - | metabolic process |
| Enolase | Magnesium ion binding | A0A077YX57 | 44.49 | 27 | 49.513 | - | glycolytic process |
| Glyceraldehyde-3-phosphate dehydrogenase | Oxidoreductase | A0A077ZHV3 | 56.10 | 66 | 37.536 | - | glycolytic process |
| Malic enzyme | Oxidoreductase | A0A077Z5U2 | 28.04 | 13 | 62.847 | - | Unknown |
| Phosphoenolpyruvate carboxykinase GTP | Kinase | A0A077Z7M0 | 29.04 | 20 | 70.975 | - | gluconeogenesis |
| Triosephosphate isomerase | Isomerase | A0A077ZC84 | 57.26 | 10 | 27.399 | - | gluconeogenesis |
| **Cytoskeleton, motility, and muscle** | | | | | | | |
| Actin | ATP binding | A0A077ZE37 | 55.59 | 35 | 41.838 | - | unknown |
| Actin 5C | ATP binding | A0A077YWW9 | 53.66 | 29 | 41.036 | - | unknown |
| Epididymal secretory protein E1 | Intracellular cholesterol transport | A0A077ZOI4 | 43.44 | 28 | 45.783 | 1 to 23 | unknown |
| Intermediate filament protein IFA 1 | Unknown function | A0A077Z6U0 | 23.39 | 14 | 70.711 | - | unknown |
| Moesin-ezrin-radixin 1 | Actin binding | A0A077ZIT0 | 25.97 | 12 | 55.989 | - | unknown |
| Paramyosin | Motor activity | A0A077Z8E1 | 38.61 | 30 | 101.488 | - | unknown |
| Tropomyosin | Unknown function | A0A077ZIM1 | 41.20 | 38 | 87.298 | - | unknown |
| **Proteolysis** | | | | | | | |
| Cytosol aminopeptidase | Aminopeptidase activity | A0A077Z3I7 | 23.80 | 10 | 54.409 | - | unknown |
| Peptidase M13 and Peptidase M13 N domain containing protein | Metalloendopeptidase activity | A0A077ZJE5 | 24.05 | 14 | 81.361 | - | unknown |
| **Signaling** | | | | | | | |
| 78 kDa glucose regulated protein | ATP binding | A0A077Z8G8 | 22.58 | 12 | 72.784 | 1 to 18 | unknown |
| CBM 14 domain containing protein | Chitin binding | A0A077Z111 | 46.72 | 38 | 95.908 | - | chitin metabolic function |
| CBM 14 domain containing protein | Chitin binding | A0A077Z8B3 | 28.37 | 18 | 78.597 | - | chitin metabolic process |
| Galectin | Carbohydrate binding | A0A077YZM7 | 50.72 | 27 | 31.967 | - | unknown |
| Galectin | Carbohydrate binding | A0A077ZG03 | 39.64 | 25 | 32.25 | - | unknown |

## Stress and detoxification

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Chaperonin protein heat shock protein 60 | ATP binding | A0A077ZIE8 | 28.00 | 11 | 62.806 | - | protein folding |
| Heat shock protein 70 | L-malate dehydrogenase activity | A0A077Z8E4 | 20.07 | 21 | 130.299 | - | stress response |
| Heat shock protein 90 | ATP binding | A0A077Z1F6 | 17.08 | 12 | 82.924 | - | protein folding |
| Protein disulfide-isomerase | Protein disulfide isomerase activity | A0A077ZJZ3 | 35.03 | 14 | 55.125 | 1 to 18 | cell redox homeostasis |
| Protein disulfide-isomerase | Protein disulfide isomerase activity | A0A077ZLF1 | 35.95 | 15 | 55.73 | 1 to 16 | cell redox homeostasis |
| Superoxide dismutase [Cu-Zn] | Oxidoreductase | A0A077Z345 | 69.86 | 12 | 15.274 | - | unknown |

## Transcription and Translation

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 40S ribosomal protein SA | Structural constituent of ribosome | A0A077YZD4 | 42.57 | 13 | 34.141 | - | ribosomal small subunit assembly, translation |
| Elongation factor 1-alpha | Elongation factor | A0A077YYL7 | 33.48 | 12 | 51.086 | - | protein biosynthesis |
| Mediator of RNA polymerase II transcription subunit 22 | Protein disulfide isomerase activity | A0A077Z2H0 | 69.06 | 17 | 15.485 | 1 to 19 | cell redox homeostasis |
| Ribosomal L18p and L18 c domain containing protein | Structural constituent of ribosome | A0A077ZPB6 | 42.67 | 13 | 35.744 | - | translation |

## Lipid binding and transport

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Vitellogenin N and VWD and DUF1943 domain containing protein | Lipid transporter activity | A0A077ZE83 | 56.35 | 205 | 198.527 | 1 to 19 | unknown |
| Uncharacterized protein | Lipid binding | A0A077ZMT5 | 14.14 | 20 | 84.314 | - | unknown |

## Others

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| DUF290 domain containing protein | Unknown function | A0A077Z8H2 | 43.67 | 20 | 17.876 | 1 to 19 | Unknown |
| Poly-cysteine and histidine tailed protein isoform 2 | Unknown function | A0A077Z5Q5 | 50.79 | 109 | 50.494 | - | Unknown |
| Protein asteroid | Unknown function | A0A077Z2C7 | 63.64 | 34 | 30.674 | 1 to 23 | Unknown |
| Transthyretin-like protein 46 | Unknown function | A0A077Z9N4 | 42.57 | 12 | 16.458 | 1 to 18 | Unknown |
| Uncharacterized protein | Unknown function | A0A077YXT2 | 16.08 | 10 | 69.581 | 1 to 18 | unknown |
| Uncharacterized protein | Unknown function | A0A077YX18 | 20.42 | 10 | 32.73 | 1 to 18 | unknown |
| Uncharacterized protein | Unknown function | A0A077Z544 | 48.83 | 19 | 33.553 | 1 to 23 | unknown |

*The number of distinct peptides having at least 95% confidence.

**Molecular function and biological process was obtained from the Gene Ontology (GO) database https://www.uniprot.org.

The most abundant category for the biological process assigned to the egg proteins were protein folding, translation, gluconeogenesis, and glycolytic process all equally represented (13%), followed by cell redox homeostasis (12%), chitin metabolic function (12%), and to a lesser extent metabolic processes, carbohydrate metabolic processes, protein biosynthesis and stress responses **(Figure** 4.4).
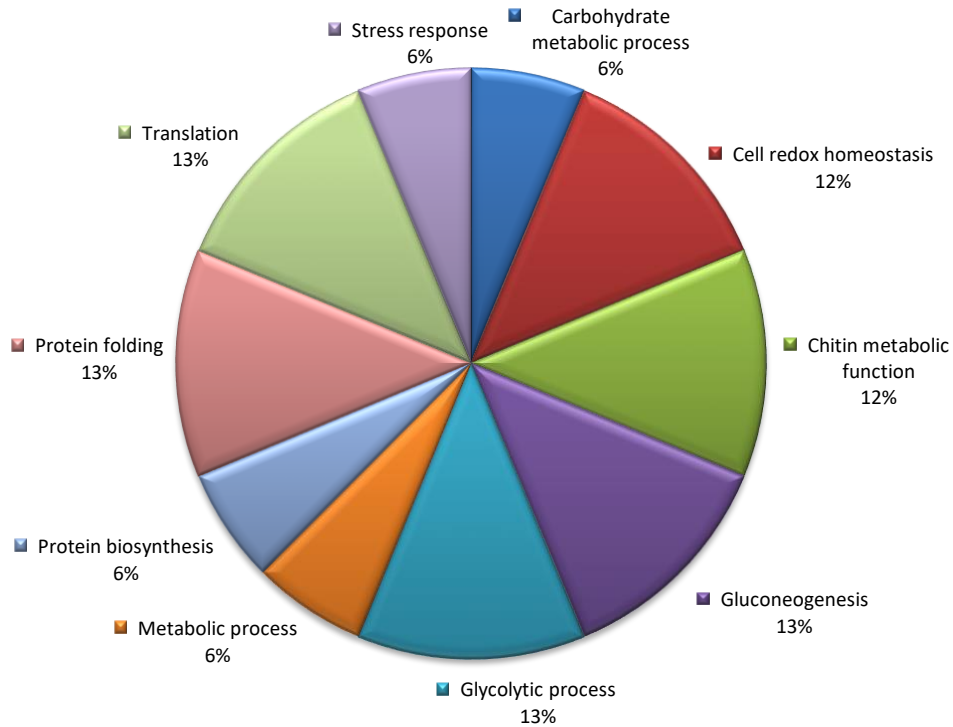


Figure 4.4 Main biological processes of the identified proteins in the non-embryonated egg extract proteome of *T. trichiura* according to information obtained from the Gene Ontology (GO) database.
https://www/uniprot.org

### 4.2.3   1-DE and immunoblot analysis of *T. trichiura* EE and FE

To identify the species-specific parasite antigens, 1-DE SDS-PAGE and Western blots were performed on the egg extract (EE) and the female extract (FE) with serum and saliva from naturally infected AGMs.

For the EE, the possible identity of the antigens revealed in Western blots (**Figure** 4.5), Lane 1 and 1.1) was investigated by matching the molecular weights of the bands seen with those of proteins identified in the EE proteome and are presented in **Table** 4.4. In addition, the same

specific areas on SDS-PAGE gels (1G and 2G, **Figure** 4.5 Lane 2) corresponding to the bands in the Western blots were excised and used for confirmatory proteomic analysis by LC-MS/MS and presented in **Table** 4.5.

The immune complexes identified by Western blot with the EE as antigen ranged from 37 to 200 kDa with the most immunogenic in two distinct bands, band 1W (≈ 170 kDa) and band 2W (≈ 37 kDa) (**Figure** 4.5, Lane 1 and 1.1).

For the FE, the comparative study for the specific immune-complexes identified by Western blot (**Figure** 4.5, lane 3 and 3.1) and areas matched on SDS-PAGE gels (**Figure** 4.5, Lane 4) were also excised and analyzed by LC-MS/MS and presented in **Table** 4.6. With the FE as antigen these ranged from 33 to 70 kDa with the most immunogenic in two distinct bands, band 3W (≈ 60 -70 kDa) (**Figure** 4.5, lane 4) and band 4W (≈ 37 kDa) (**Figure** 4.5, lane 3 and 3.1).
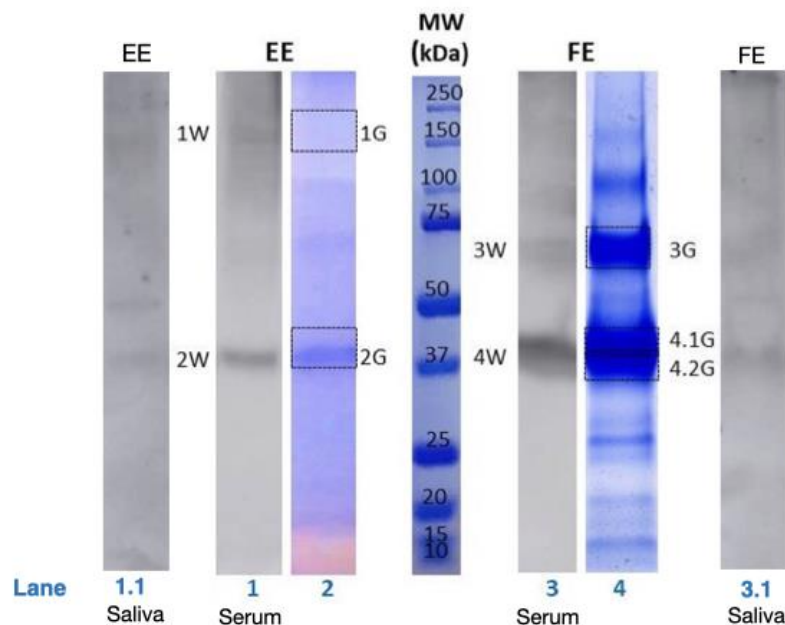


**Figure 4.5 Major immunogenic proteins detected in *T. trichiura* extracts.**
Western blot showing AGMs sera and saliva antibodies response to *T. trichiura* egg extract (EE) (lane 1 and 1.1) and female extract (FE) (lane 3 and 3.1) (10 µg/lane). Saliva or serum samples diluted 1:10 or 1:500, respectively. Secondary antibody (peroxidase-labeled goat anti-primate IgG (1:5000) or IgA (1:1000).

Bands 1W-2W and 3W-4W indicate the regions containing antigens recognized most strongly by sera and salivary antibodies in EE and FE, respectively. Corresponding SDS-PAGE of EE (lane 2) and FE (lane 4), stained with Coomassie Brilliant Blue R-250 and excised areas of each, 1G-2G and 3G-4.1G-4.2G, containing the most immunogenic peptides for proteomic analysis. Molecular weight in kDa is lane labeled as MW.

**Table 4.4 Potential identity of the EE proteins targeted by serum and salivary antibodies based on the MW data of the EE proteome.**

| Accession number | Annotation | MW (kDa) | Peptides* (95%) |
|---|---|---|---|
| **Band 1W (≈ 170 kDa)** | | | |
| A0A077ZE83 | Vitellogenin N and VWD and DUF1943 domain containing protein | 198.527 | 205 |
| A0A077Z8E4 | Heat shock protein 70 | 130.299 | 21 |
| **Band 2W (≈ 37 kDa)** | | | |
| A0A077Z5Q5 | Poly-cysteine and histidine tailed protein isoform 2 | 50.494 | 109 |
| A0A077ZHV3 | Glyceraldehyde-3-phosphate dehydrogenase | 37.536 | 66 |
| A0A077ZE37 | Actin | 41.838 | 35 |
| A0A077YWW9 | Actin 5C | 41.036 | 29 |
| A0A077YX57 | Enolase | 49.513 | 27 |
| A0A077Z0I4 | Epididymal secretory protein E1 | 45.783 | 28 |
| A0A077Z8B3 | CBM 14 domain containing protein | 78.597 | 18 |

*The number of distinct peptides having at least 95% confidence.

Based on corresponding molecular weights in the EE proteome, band 1W contained vitellogenin N and VWD and DUF1943 domain containing protein (VgNVD) with 205 distinct peptides and heat shock protein 70 (HSP-70) with 21 distinct peptides (**Table** 4.4). Confirmatory LC-MS/MS of the corresponding Coomassie-stained band confirmed that VgNVD, with 241 distinct peptides, was the most representative protein within the 1W area (**Table** 4.5).

Analysis of band 2W identified poly-cysteine and histidine-tailed protein isoform 2 (PCHTP-2), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), actin, actin 5C, enolase, epididymal secretory protein E1, and CBM 14 domain containing protein (CBM14) (**Table** 4.5) and confirmatory analysis with LC-MS/MS also indicating there were other proteins present such as phosphoglycerate kinase, the dolichyl-diphosphooligosaccharide-protein glycosyltransferase 48 kDa subunit, adenosylhomocysteinase, tubulointerstitial nephritis antigen, calponin domain

containing protein, 3 ketoacyl coenzyme A thiolase, elongation factor 1-alpha and serpin domain containing protein (**Table** 4.5) while actin 5C and epididymal secretory protein E1 were not confirmed.

Table 4.5 Protein identities, in decreasing abundance, in immunodominant bands 1W and 2W in Western blots with EE as antigen. Proteins were identified by LC-MS/MS of corresponding areas in SDS-PAGE gels, 1G and 2G.

| Accession number | Annotation | MW (kDa) | Peptides* (95%) |
|---|---|---|---|
| Area 1G (≈ 150-200 kDa) | | | |
| A0A077ZE83 | Vitellogenin N and VWD and DUF1943 domain containing protein | 198.527 | 241 |
| A0A077Z8E4 | Heat shock protein 70 | 130.299 | 3 |
| Area 2G (≈ 37-45 kDa) | | | |
| A0A077Z5Q5 | Poly-cysteine and histidine tailed protein isoform 2 | 50.940 | 66 |
| A0A077Z8B3 | CBM 14 domain containing protein | 78.597 | 24 |
| A0A077YX57 | Enolase | 49.513 | 18 |
| A0A077ZHV3 | Glyceraldehyde-3-phosphate dehydrogenase | 37.536 | 15 |
| A0A077ZE37 | Actin | 41.838 | 14 |
| A0A077Z3K7 | Phosphoglycerate kinase | 44.724 | 12 |
| A0A077Z8X2 | Dolichyl- diphosphooligosaccharide-protein glycosyltransferase 48 kDa subunit | 48.787 | 12 |
| A0A077ZF21 | Adenosylhomocysteinase | 47.827 | 10 |
| A0A077Z3H9 | Tubulointerstitial nephritis antigen | 50.458 | 9 |
| A0A077YXR0 | Calponin domain containing protein | 40.694 | 7 |
| A0A077ZJA3 | 3 ketoacyl coenzyme A thiolase | 43.402 | 6 |
| A0A077YYL7 | Elongation factor 1-alpha | 51.086 | 6 |
| A0A077Z1Z4 | Serpin domain containing protein | 43.328 | 6 |

*The number of distinct peptides having at least 95% confidence.

Regarding the comparative proteomic analysis of reactive areas displayed in FE Western blot, the analysis of the band 3G (≈ 60 -70 kDa) (**Figure** 4.5, lane 4), which corresponded to band 3W, revealed again PCHTP-2 as one of the proteins identified with the highest number of matching peptides. This protein was also identified in bands, 4.1G and 4.2G (**Table** 4.6). The proteomic results of both sections of the band 4W showed some proteins shared between the EE and FE, such as PCHTP-2, Actin and GAPDH, suggesting they are likely to be the major ones in both samples. This finding is not surprising, since most of the EE antigens are also present in FE (eggs contained in the uterus).

Table 4.6 Protein identities, in decreasing abundance within FE excised gel areas (3G, 4.1G, 4.2G) with suitable MW matching Western blot band areas 3W and 4W.

| Accession number | Annotation | MW (kDa) | Peptides* (95%) |
|---|---|---|---|
| Area 3G (≈ 60-70 kDa) | | | |
| A0A077ZIM1 | Tropomyosin | 87.298 | 19 |
| A0A077ZIM7 | Papilin | 80.804 | 6 |
| A0A077ZEY0 | Calsequestrin | 49.211 | 5 |
| A0A077YX57 | Enolase | 40.513 | 5 |
| A0A077Z9R9 | Kunitz BPTI domain containing protein | 103.388 | 2 |
| Area 4.1G (≈ 37-45 kDa) | | | |
| A0A077Z5Q5 | Poly-cysteine and histidine tailed protein isoform 2 | 50.494 | 65 |
| A0A077ZE37 | Actin | 41.838 | 7 |
| A0A077ZHV3 | Glyceraldehyde-3-phosphate dehydrogenase | 37.536 | 5 |
| A0A077ZEY0 | Calsequestrin | 49.211 | 5 |
| Area 4.2G (≈ 33-37 kDa) | | | |
| A0A077Z0I4 | Epididymal secretory protein E1 | 45.783 | 8 |
| A0A077Z0N1 | Actin-depolymerizing factor 2, isoform c | 35.411 | 4 |
| A0A077Z5Q5 | Poly-cysteine and histidine tailed protein isoform 2 | 50.494 | 4 |

*The number of distinct peptides having at least 95% confidence.

In comparing the Western blots with EE and FE as antigen, highly reactive bands were seen in both with a molecular weight of around 37 kDa (2W and 4W, **Figure** 4.5, lane 1 and 3). There were intensely staining protein bands in the corresponding SDS-PAGE gels. The other two prominent bands seen in the Western blots of the EE and FE antigens, 1W and 3W, were of different molecular weights and thus seemed to be stage-specific to the different life stages (**Figure** 4.5). Antigenic band 1W was barely detected in the corresponding SDS-PAGE indicating a low concentration of protein, while band 3W corresponded to a prominent band on the corresponding SDS-PAGE gel indicating a high concentration (3G) (**Figure** 4.5, Lane 4). Although the 2W and 4W major antigenic bands of the EE and FE, respectively, both had a molecular weight of 37 kDa and shared several proteins (PCHTP-2, Actin and GAPDH), there were also specific proteins which appeared in only the egg or adult female stage. Dolichyl-diphosphooligosaccharide-protein glycosyltransferase 48kDa subunit, CBM14 domain containing protein, adenosylhomocysteinase, tubulointerstitial nephritis antigen, calponin domain containing protein, 3 ketoacyl coenzyme A thiolase, elongation factor 1-alpha and serpin domain containing protein

appeared as typical of EE (**Table** 4.5). Meanwhile, Calsequestrin, Epididymal secretory protein E1, Actin-depolymerizing factor 2-isoform c and Kunitz Bovine basic pancreatic trypsin inhibitor (BPTI) domain containing protein (KBDCP) were only present in FE (**Table** 4.6).

## 4.3 Identification of immunogenic proteins of diagnostic value

### 4.3.1 Specificity Immunoblot analysis

To identify patterns of parasite antigenic responses across different species, Western blots were performed on extracts of other helminths, nematodes (*Primasubulura* sp., *Anisakis simplex, Aspiculuris tetrapter*a) and trematodes (*Fasciola hepatic*a, *Dicrocoelium dendrit*icum and *Gnathosoma hispidium*) with serum from naturally infected AGMs.

The immune complexes identified by Western blot with the *Primasubulura* sp., *A. simplex* and *A. tetrapter*a as antigen ranged from 37 to 250 kDa with the most immunogenic band ($\approx$ 50 kDa) for *Primasubulura* sp. and *A. simplex*, and ($\approx$ 150 kDa) *A. tetrapter*a (**Figure** 4.6). *Fasciola hepatica* immune-complex identified was a pronounced single band ($\approx$ 100 kDa) while *D. dendrit*icum immune-complexes were mildly reactive (**Figure** 4.6). *Gnathosoma hispidium* immune complexes presented the widest range from 15 to 100 kDa with the most immunogenic distinct bands at ($\approx$ 15 kDa, $\approx$ 25 kDa, $\approx$ 50 kDa, $\approx$ 75 kDa, $\approx$ 100 kDa) (**Figure** 4.6).
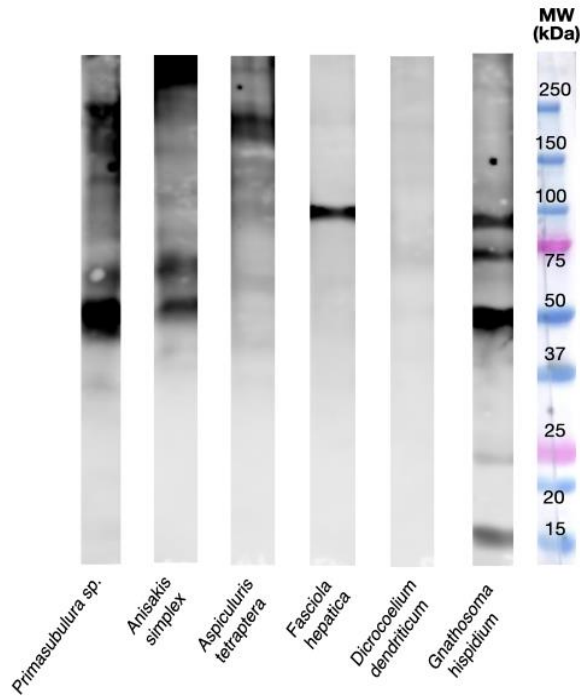
Figure 4.6 Western blot showing AGMs sera antibodies in nematodes and trematodes extracts. Molecular weight in kDa. Western blot showing AGMs sera antibodies response in nematodes and trematodes extracts (10 μg/lane). Serum samples diluted 1:500, secondary antibody (peroxidase-labeled goat anti-primate IgG (1:5000).

### 4.3.2 2-DE SDS-PAGE electrophoresis and Western-Blot with serum antigenic profile

To confirm the most reactive immune complexes MW zones 2-DE SDS-PAGE and Western blot were performed on EE with serum from naturally infected AGMs. The possible MW identity of the antigens revealed in Western blots (**Figure** 4.7, zone 1 (Z1), 2 (Z2) and 3 (Z3)) was investigated by matching the molecular weights of the zones observed in the EE 2-DE SDS-PAGE (**Figure** 4.7).

The immune-complexes identified by Western blot with the EE as antigen ranged from 50 to 200 kDa with the most immunogenic zones, zone 1 (Z1) (≈ 50 - 150 kDa; pI 5-7), zone 2 (Z2) (≈ 50 kDa; pI 7-8) and zone 3 (Z3) (≈ 50 - 200 kDa; pI 8-10) (**Figure** 4.7).
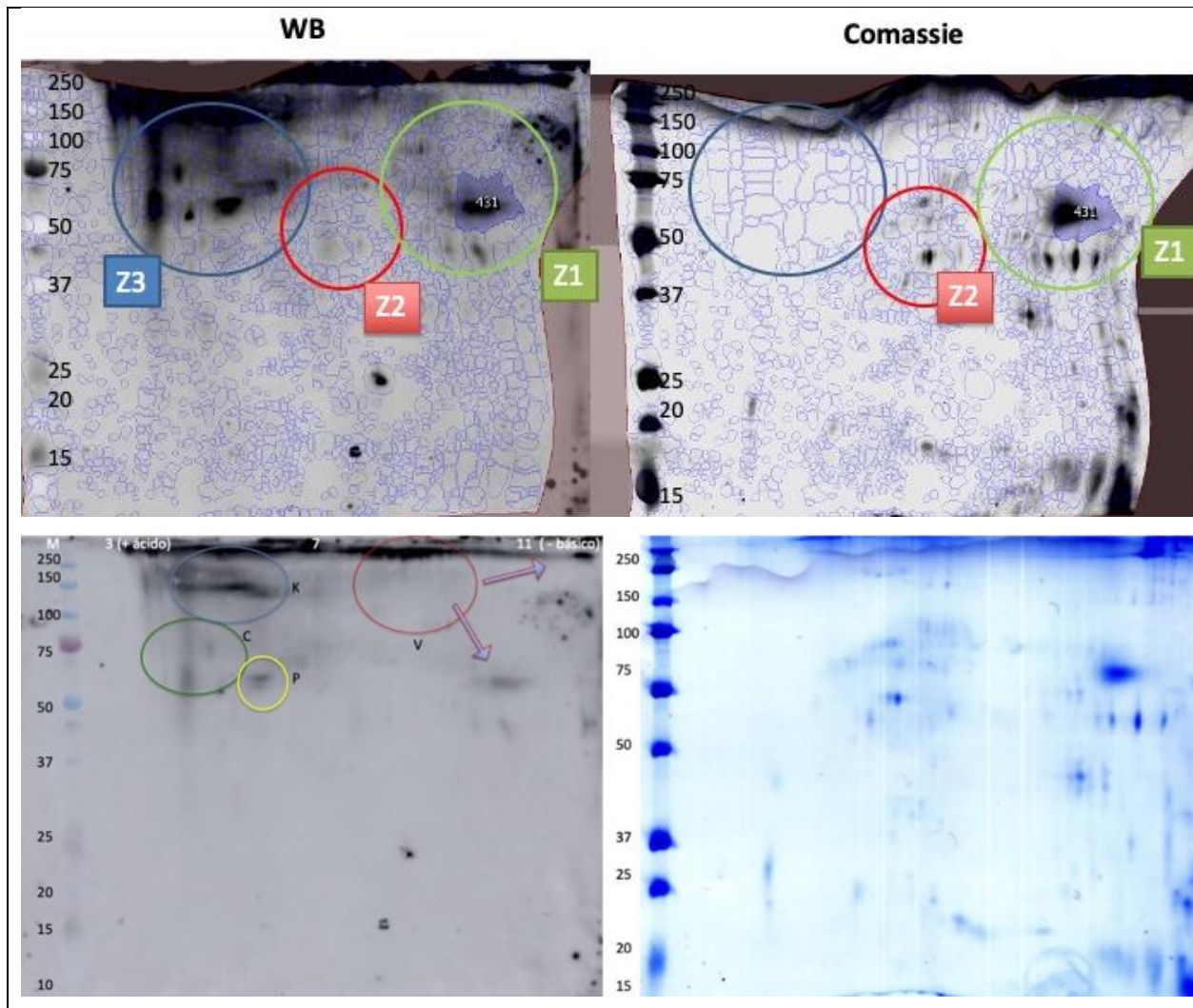
Figure 4.7 2-DE SDS-PAGE electrophoresis and Western-Blot with serum. Antigenic profile detected in *T. trichiura* egg extracts (EE).

Western blot showing AGMs sera antibody response to *T. trichiura* egg extract (EE). Egg Extract 10 μg for Coomassie staining, and 30 μg for 2-DE Immunoblotting. Serum was diluted 1:500 and secondary antibody (peroxidase-labeled goat anti-primate) IgG diluted 1:5000.

Zones 1, 2 and 3 indicate the regions containing antigens recognized most strongly by sera antibodies in EE. Corresponding SDS-PAGE of EE stained with Coomassie Brilliant Blue R-250. Molecular weight in kDa.

## 4.4 Selection and evaluation of applicability of designed synthetic peptides

## 4.4.1 Final protein candidates and derived peptide selection

### 4.4.1.1 Vitellogenin N and VWD and DUF1943 domain containing protein

| Vitellogenin N and VWD and DUF1943 domain containing protein [Acc A0A077ZE83] | | | | | | |
|---|---|---|---|---|---|---|
| Peptides | Location and Length of aa residues | Antigenicity Score | Percentage of homology with *T. trichiura* | E -value *T. trichiura* | Percentage homology to other nematode | E - value other nematode |
| YRSLAIDDVLTETAP* | 15 aa @ 1608-1622 | 1.119 | 100 % | 4e-06 | 100 % *T. suis* | 4e-06 |
| QFISPCPRCHQLP | 13 aa @ 254-266 | 1.162 | 100 % | 1e-05 | 77 % *T. suis* | 0.17 |

*Selected peptide highlighted

Selected peptide section: YRSLAIDDVLTETAP, 15 aa, from 1608 - 1622

Accession number UniProtKB: A0A077ZE83

Gene: TTRE_0000643201

MW: 196.58 kDa

Theoretical pI: 8.61

Molecular function: Lipid transporter activity

Enables the directed movement of lipids into, out of or within a cell and/or between cells.

Biological process: unknown

Subcellular location: unknown

Signal peptide: 1 – 19

Chain: 20 – 1709

Family & Domains:

1. Vitellogenin domain: 42 – 702 (661 aa)

2. Domain of unknown function (DUF) DUF1943: 742 – 1026 (285 aa)

3. Von Willebrand factor type D domain (VWFD): 1358 – 1513 (156 aa)

**Pfam model:** <span><u>http://pfam.xfam.org/protein/A0A077ZE83</u></span> accessed April 9, 2021
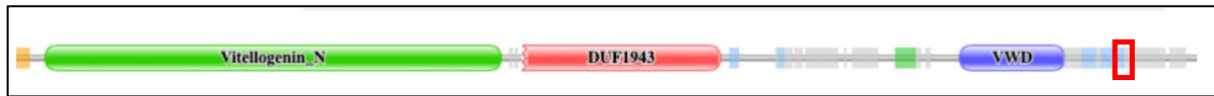


Signal peptide in orange, domains labeled in color, gray areas are disordered areas, light blue areas are low complexity areas and small green area is the coiled coil. Selected peptide section outlined in red: **YRSLAIDDVLTETAP**, 15 aa, from 1608 – 1622.

### Vitellinogen domain:

Vitellinogens are post-translationally glycosylated and phosphorylated in the endoplasmic reticulum and Golgi complex before being secreted to be taken up by oocytes. In the ovary, vitellinogens bind to specific Vtgr receptors on oocyte membranes to become internalized by endocytosis, where they are cleaved into yolk proteins by cathepsin D (<u>http://www.ebi.ac.uk/interpro/</u>, accessed April 14, 2021).

### Domain of unknown function (DUF) DUF1943:

This domain adopts a structure consisting of several large open beta-sheets and their exact function has not been determined (<u>http://www.ebi.ac.uk/interpro/</u>, accessed April 14, 2021).

### Von Willebrand factor, type D domain

Von Willebrand factor (VWF) is a large, multidomain blood glycoprotein synthesized in endothelial cells and megakaryocytes, that is required for normal hemostasis. VWF mediates the adhesion of platelets to sites of vascular damage by binding to specific platelet membrane glycoproteins and to constituents of exposed connective tissue. It is also essential for the transport of the blood clotting factor VIII (<u>http://www.ebi.ac.uk/interpro/</u>, accessed April 14, 2021).

**FASTA** sequence: signal peptide and selected peptide in bold

**MGLKVVILTLLAFALYAAG**RHQQRLKLLRDTVNYQNVQESYFRINQKYKFKYNGQVKIGV
PDHSNQNSMTRFTAEVTLVKRSEEHFIIRVNNIRLGKQLGNSKDQDEMASFEELEPVEIK
QSDLKILELPVEFTYAEGQVQDLVFQQDDEEWSENLKRGIINLFQIKLQSTDRTSMEEEQ
SALNRIDTESKTNVGTAYRTREKTVEGECDTMYTVSAIDDDDDDNSERGSRMLVTKAIDM

KSCQRRPEVWWNFQFISPCPRCHQLPRSGERSVESSTTIRYQIRGKRDKFLIERVELSND
HVIAQQNADESAVVVKIKASLKLVSSQESNDGSSEMSNFPATGQRVSDLIYSTRDDEHFD
RFYAEGDQHYNDRLFSRRQKGQDKSAALAEIIMKMMRHMKDTADEKANRYFYKAVQLMRY
MSESEIRSTNEHHFGRQQSGMLTPEERERARNIMPNLLAQAGTLSSFRQLADKIANGEIN
PLKAAIVITTMMDTPRVSKEIITELMRLDESQTVQRNEQLRRAILLTAGSMMRTMCAPQR
HQRQQRQESQNRDDIRTDNSNHQRCDSEIKQRFVRTIADRLAASDRWEDQVILIRALGNA
GLDVSISELESIIRNQDRRNPAAIRLEAILALRHIKDSLPQKTKNILLSAAANRMESSAV
RMAAIQMLLQQNPDRMTIDQIGVIINHDPNRRVASFAYKLIRRLADSNQPCYEENKQKLQ
TVAKSVRRRNLQLPHSDMIFESVYDREKKTGFDFFIMPMYDMDDIVPKFMRAGINMVERG
ERNRNLFALEIGTSSLSNIISELLPDIEQNQRGGNTEIKMKLRRMAEETRRNGNRGSRQQ
QQQRQQQQRQTKAWMSMKFRNQDIMLLTFNEDRLKQLTQQNRGAESLLLLIAKMASNRQG
SISIDEATLLRETVVKIPTAIGSQLSIRRKAPAIFSAHGQASIGRNIPIQADIRARISTT
ISMVTDVSSRTPISTNGIYLIKNIKATVPVDMTISLDHRQEDEQLKIQMRNHEGYKDLLK
LESRPVIYYMRSQNPLAEAEEKTVVAEKNVRMESFKRCMRGPLFGSEICMRGVITTPMCN
HNKLLHYMAPWFGPNKVTISAYMRRTEENENENENSRQVGLTIRSDSMQNKLELEYETSS
RSLIPTKVDAKLQRQGHDGQRQEICINMKTQGKENDQRSEQTRRSSDININWGTQCNDEN
YIKARIETASRRNVIWEQRQSNAIQEDNEERDQKQIGYGVSSEETAGYKIQIQHRNVPEW
AVDKAEDIIRMLTSMNYWSTEIENKRQRYDSAERNRQRDEQSRAGEVRIQAIMRNEDKAD
VKIQTPRKTIRMNNVYIPTLLRKETYRRSNFMRLMNLYMGNKHAKGTCQIRQNSITTFDG
AIYRIPFSSCYTILARDSEEDPKFAIMARRSREQPDKKVVKLMTSDHEIELIPERGGGIE
VKVDGQRWDDQQSKHHRAMRIRKRENEVTVDLKRPNVDVHYDGNEITIRVSDKYHGRQTG
MCGNLNADSSDEFSKSNQKNRDDIWETFNEMTIRTDDCQHPQRQEQDDSQQFDSYDSDSD
SNSYDSWEMARQHRSDDSISYDDEEESSMFDSDQQQQQRRDQERNQH**YRSLAIDDVLTET**
**AP**VRRNKVIDSRGRKCISMRPIESCPEHGYTARGEKEEKEVPYTCLKANDELYHKIGRMQ
RQGTPMDLSNSEMSFTRKETVPRKCISIV

## 3D structure databases:

1. **SWISS model EXPASY:**

   LIPOVITELLIN (LV-1N, LV-1C) 1ish.1.A (Identity: 17.21) and LIPOVITELLIN (LV-2) 1ish.1.B
   (Identity: 13.36) (https://swissmodel.expasy.org/interactive/NTnrVq/templates/).
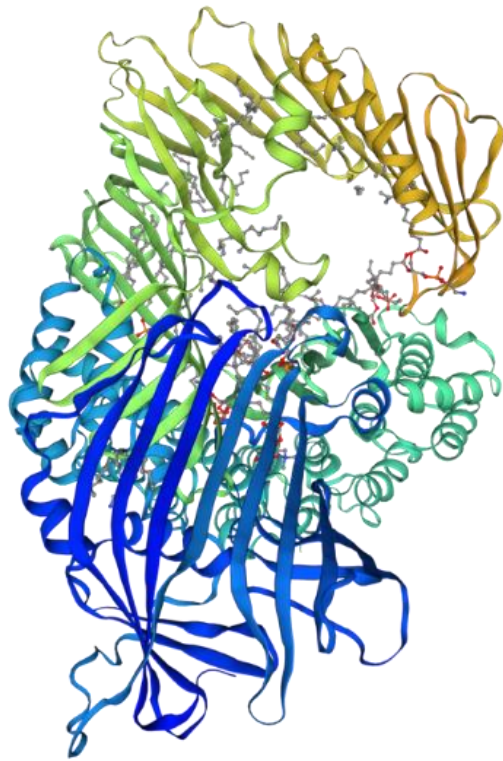
Figure 4.8 3D model Vitellogenin N and VWD and DUF1943 domain containing protein

*Model based on known protein identities
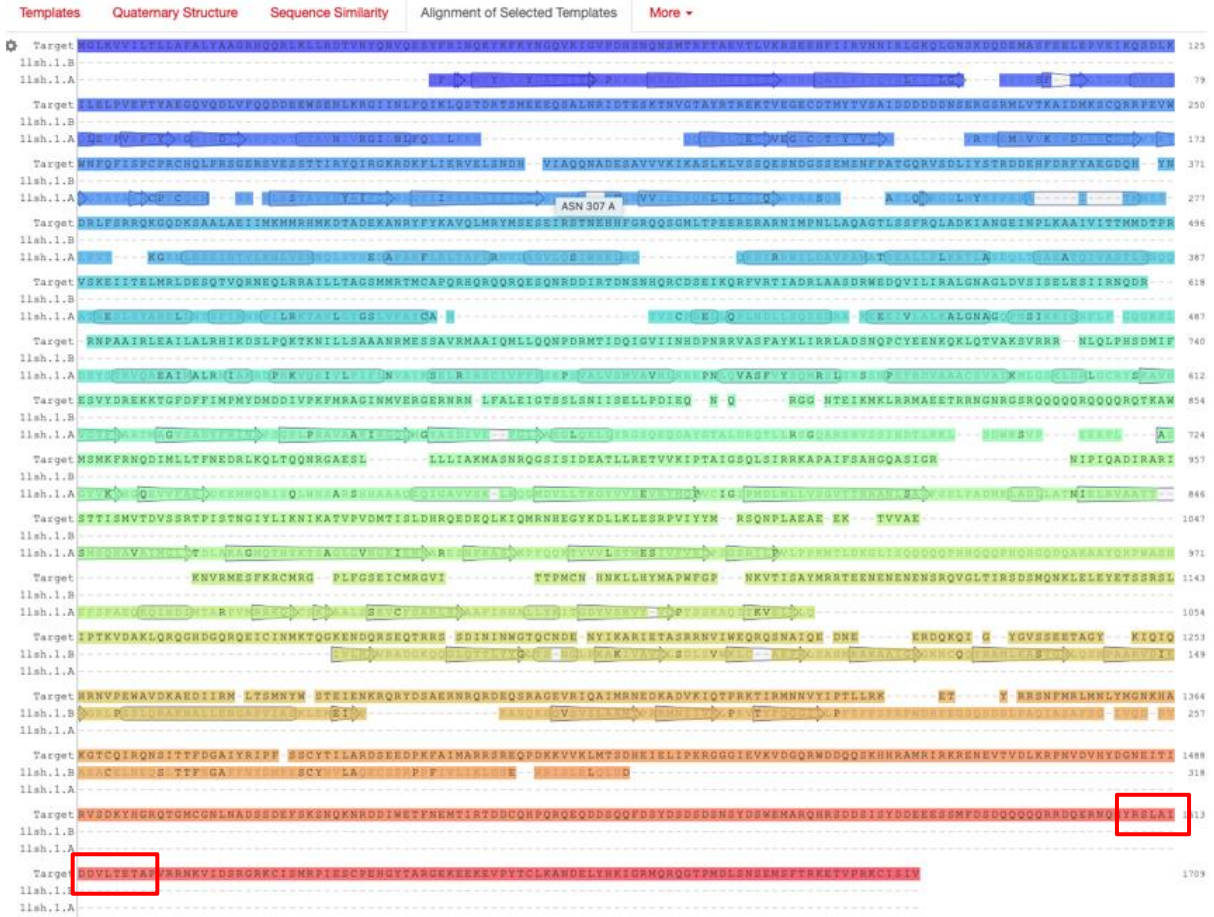
**Figure 4.9 Alignment of known protein identities.**

Selected peptide outlined in red: **YRSLAIDDVLTETAP**, 15 aa, from 1608 – 1622.

## 4.4.1.2 CBM 14 domain containing protein

| | | | | | | |
|---|---|---|---|---|---|---|
| **CBM 14 domain containing protein [Acc A0A077Z8B3]** | | | | | | |
| Peptides | Location and Length of aa residues | Antigenicity Score | Percentage of homology with *T. trichiura* | E -value *T. trichiura* | Percentage of homology to other nematode | E - value other nematode |
| <mark>SAPTTVTTQLSPVDCVTL</mark> | 18 aa @ 230-247 | 1.196 | 100 % | 9e-09 | 78% *T. suis* | 8e-04 |
| RVTVTSCELQFSLRHF | 16 aa @392-407 | 1.126 | 100 % | 7e-08 | 56% *Acidobacteria bacterium* | 13 |

*Selected peptide highlighted

Our selected peptide section: SAPTTVTTQLSPVDCVTL, 18 aa, from 230 - 247

Accession number UniProtKB: A0A077Z8B3

Gene: TTRE_0000321001

MW: 78.597 kDa Theoretical pI: 5.20

**Molecular function:** chitin binding

Interacting selectively and non-covalently with chitin, a linear polysaccharide consisting of beta-(1 - > 4)- linked N-acetyl-D-glucosamine residues.

**Biological process:** chitin metabolic process

**Subcellular location:** extracellular region

**PTM/Processing:** none

**Family & Domains:** 9 Chitin binding Peritrophin-A domains

1. CBM 14 domain: 10 – 67 chitin-binding type 2.

2. CBM 14 domain: 84 – 144 chitin-binding type 2.

3. CBM 14 domain: 164 – 223 chitin-binding type 2.

4. CBM 14 domain: 241 – 300 chitin-binding type 2.

5. CBM 14 domain: 322 - 381 chitin-binding type 2.

6. CBM 14 domain: 418 – 477 chitin-binding type 2.

7. CBM 14 domain: 496 – 555 chitin-binding type 2.

8. CBM 14 domain: 576 – 633 chitin-binding type 2.

9. CBM 14 domain: 653 – 713 chitin-binding type 2.

**Pfam model:** showing the 9 CBM 14 domains in green and low complexity areas in light blue.

http://pfam.xfam.org/protein/A0A077Z8B3 accessed April 14, 2021
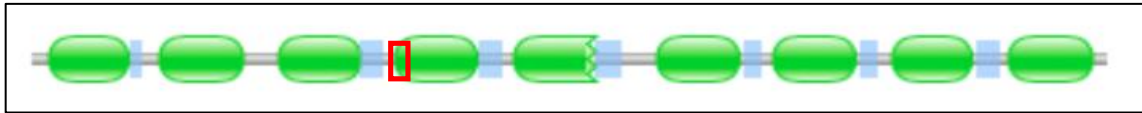


Selected peptide outlined in red **SAPTTVTTQLSPVDCVTL**, 18 aa, from 230 – 247

**Chitin binding Peritrophin-A domains:**

These domains are found in chitin binding proteins particularly peritrophic matrix proteins of insects and animal chitinases. It is an extracellular domain that contains six conserved cysteines (motif) that potentially form three disulfide bridges (http://www.ebi.ac.uk/interpro/, accessed April 14, 2021)

**FASTA** sequence: selected peptide in bold

MGSMKLIDFLEKCAHIPDGNYPISPCSHHYLACSGGRGIIRVCPSGLIYNPVRNRCDNKA
DVATCGVSTTTTTTPLSDITKQQTFDCSGLLPGDYPLQANSCLQQYFTCDANSVGVVRTC
PNDLYFDSVNRVCNFFDNIASCSGVTAPQTTVTSTLGPTLPPVEFDCSKRKDGFYPNPAR
QCSSIYYACTAGEARRLFCGSGLAYDIVTRACQSPDNTFACTGRTTPVT**SAPTTVTTQLS
PVDCVTL**PNGLYPNPEDACSRIFFACSDGIADRFVCPGELYFDSDTQSCQRFSDVFACTG
VTTTTTTTTSASTTTTPQSDVGFDCTSLADGLYPNPNEICSTFYFICSGSVARRQNCPSG
LYYDPEIQRCNSFGNIFVCTGTRPTTTTTTTRVTVTSCELQFSLRHFECNMPKLSAPDLD
CTNLANGLYPNPRSQCSPIYFYCTNGFAYERRCLDDLFFNPELKICDRYSDIFECTGTRS
TPTTTPTPTTPQSDVPFNCAYLPNGNYPNPSQQCSNMFFTCSNGKATIRTCPQETYFDPE
LRLCLKFSDVPACSGTQRTTTTATTTTALSDVTTPFFQCGNMPNGNYPLGSCENVYVSCV
DGNPSRRECPLNLFYDYTINECDYIYRIPGCGGQRTTTVLSDVTVTTTTPAPGFDCSRLA
DGKYPNPDNVCYSDFYYVCAGGRTSRMYCPAGLIYDPNAQECRFRRHAKPCKKSSLGSL

**3D structure databases:**

1.  **SWISS model EXPASY:**

    Carbohydrate-binding module family 14 protein, 4z4a.1.A (Identity: 37.50), dust mite allergen, 4z4a.1.B (Identity: 19.57), chitotriosidase-1, 5hbf.1.A (Identity: 22.92). (https://swissmodel.expasy.org/interactive/F5LQzS/templates/).
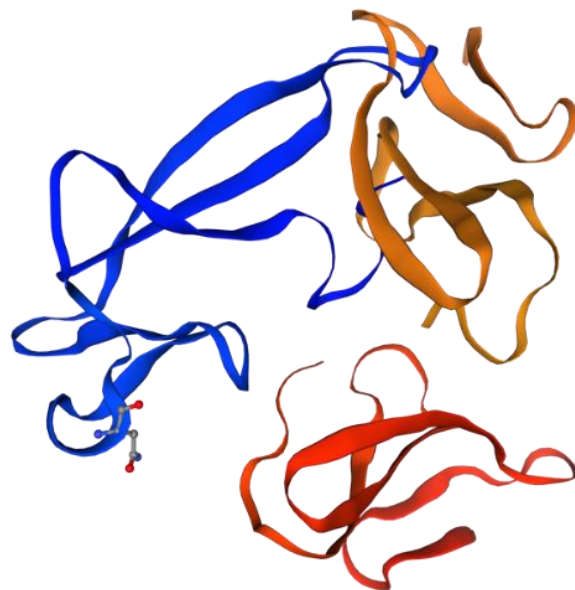
Figure 4.10 3D model CBM 14 domain containing protein model.

*Model based on known protein identities



Figure 4.11 Alignment of known protein identities.

Selected peptide outlined in red: **SAPTTVTTQLSPVDCVTL**, 18 aa, from 230 – 247.

### 4.4.1.3 Kunitz BPTI domain containing protein

| Kunitz BPTI domain containing protein [Acc A0A077Z9R9] | | | | | | |
|---|---|---|---|---|---|---|
| Peptides | Location and Length of aa residues | Antigenicity Score | Percentage of homology with *T. trichiura* | E -value *T. trichiura* | Percentage of homology to other nematode | E –value other nematode |
| QERCVATLPASVICRLP | 17 aa @ 816-832 | 1.191 | 100% | 1e-08 | 94% *T. suis* | 1e-07 |
| QQRCIATLPAIAFTL | 15 aa @ 489-503 | 1.135 | 100% | 2e-06 | 90% *T. suis* | 5.5 |

*Selected peptide highlighted

Our selected peptide section: QERCVATLPASVICRLP, 17 aa, from $816 - 832$.

Accession number UniProtKB: A0A077Z9R9

Gene: TTRE_0000541401

MW: 100.184 kDa

Theoretical pI: 5.91

Molecular function: chitin binding and serine endopeptidase inhibitor activity

Interacting selectively and non-covalently with chitin, a linear polysaccharide consisting of beta-(1 - > 4)- linked N-acetyl-D-glucosamine residues.

Stops, prevents, or reduces the activity of serine-type endopeptidases, enzymes that catalyze the hydrolysis of nonterminal peptide bonds in a polypeptide chain; a serine residue and a histidine residue are at the active center of the enzyme.

Biological process: chitin metabolic process

Subcellular location: extracellular region or secreted

PTM/Processing:

Signal peptide: 1 - 29.

Chain: $30 - 909$.

Family & Domains:

1. BPTI/Kunitz inhibitor domain: 36-86

2. BPTI/Kunitz inhibitor domain: 96-146

3. Chitin-binding type-2: 102-167

4. BPTI/Kunitz inhibitor domain: 156-206

5. BPTI/Kunitz inhibitor domain: 216-266

6. BPTI/Kunitz inhibitor domain: 276-326

7. Chitin-binding type-2: 282-350

8. BPTI/Kunitz inhibitor domain: 339-389

9. BPTI/Kunitz inhibitor domain: 385-439

10. BPTI/Kunitz inhibitor domain: 446-492

11. BPTI/Kunitz inhibitor domain: 509-559

12. BPTI/Kunitz inhibitor domain: 583-633

13. BPTI/Kunitz inhibitor domain: 645-695

14. BPTI/Kunitz inhibitor domain: 710-760

15. Chitin-binding type-2: 716-780

16. BPTI/Kunitz inhibitor domain: 769-819

17. BPTI/Kunitz inhibitor domain: 829-879

**Pfam model:** http://pfam.xfam.org/protein/A0A077Z9R9 accessed April 14, 2021

Showing the signal peptide in orange, the different domains in green and low complexity areas in light blue.
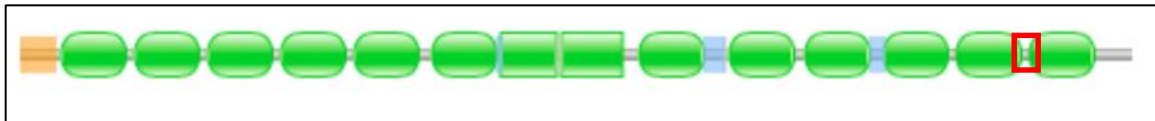


Selected peptide outlined in red **QERCVATLPASVICRLP**, 17 aa, from 816 – 832.

**Bovine pancreatic trypsin inhibitor (BPTI)/Kunitz inhibitor domain:**

They inhibit proteases and their structure is a disulfide rich alpha + beta fold. The fold is constrained by 3 disulfide bonds. The pancreatic trypsin inhibitor (Kunitz) family is one of the numerous families of serine proteinase inhibitors (http://www.ebi.ac.uk/interpro/, accessed April 14, 2021).

**FASTA** sequence: signal peptide and selected peptide in bold

**MKPHSQASGVAWRMKITVILFLLFGATFA**SRRKSPCNLTVDGGPCKAQFTKYYYNKESKK

CEPFVYGGCQGNRNRFDTLEECTAKCAEQKKQEDSCKLPAETGPCKASFTRYYYVWDQKK

CAPFTYGGCKGNANNYESIAECEEKCGVRNKDKNPCELPAETGPCMASFIRFYYNKETKK

CETFTYGGCQGNENNFETLEECEAKCSDQKKQQNPCKMGPDPGPCRAVFLRYYYSWDWKK

CLKFTYGGCEGNANNFESLAQCEATCVDKKKKDNPCELPAEPGPCKASFLRYYYNKESKK

CETFTYGGCEGNENSFETLKECEARCVKNKKRKRQETPCELPADTGPCKMNMIRYYYDKG

SKKCKTFTYGGCHGNENNFESMEKCEATCEEGEEKEEEEQGPCRAAFTKYYYNKDTKQCE

TFIYGGCRGNANNFETKAESPVEEPPIDVGPPTSHRKWFFDTVKKVCRTFVYGGCKGNGN

RFDTKEECQQRCIATLPAIAFTLQEENPCKLPADPGPCMASFIKHYYDWDSKQCLEFTYG

GCEGNANNFETIEECQKSCPGEEQEEKEEEEEEKEEEQGGNPCELPAKKGPCRAAFTKYY

YNKDTKQCETFIYGGCKGNANNFETIEECQKSCPEKEEEQGGNPCELPAKKGPCRAAFTK

YYYNKDTKQCETFIYGGCKGNANNFETIEECQESCPGEEEEEEEEQGGNPCELPAKKGPCR

AAFTKYYYNKDTKQCETFIYGGCKGNANNFETKAECESRCSVEKDGDVCSMPKDSGPCKA

SHRKWFFDAVKKVCRTFVYGGCKGNGNRFDTKEEC**QERCVATLPASVICRLP**YAMGSCSS

NLRRFFFDFETKQCRSFTYTGCEGNANNFISAYACYEKCGKRIYGNVEWCAVLIRWERQK

ASYFQSLKF


**3D structure databases:**

1. **SWISS model EXPASY:** 9 sections of Kunitz-type protease inhibitor 1 (5h7v.1.a) selected to build model (https://swissmodel.expasy.org).
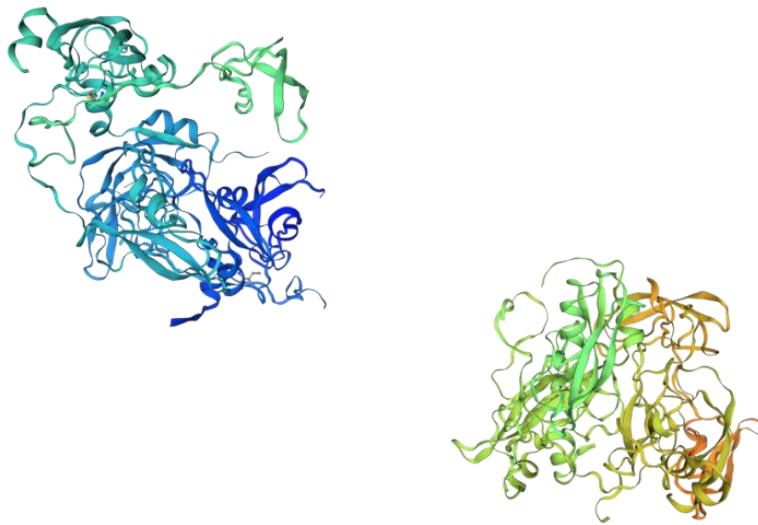


**Figure 4.12 3D model Kunitz BPTI domain containing protein.**
*Model based on known protein identities

Figure 4.13 Alignment of known protein identities.

Selected peptide outlined in red: **QERCVATLPASVICRLP**, 17 aa, from 816 − 832

### 4.4.1.4 Poly-cysteine and histidine tailed protein isoform 2

| Poly-cysteine and histidine tailed protein isoform 2 [Acc A0A077Z5Q5] | | | | | | |
|---|---|---|---|---|---|---|
| Peptides | Location and Length of aa residues | Antigenicity Score | Percentage of homology with *T. trichiura* | E -value *T. trichiura* | Percentage of homology to other nematode | E –value other nematode |
| TECVKPPAHDCPAFG | 15 aa @214-228 | 1.146 | 100% | 5e-07 | 100% *T. suis* | 5e-07 |
| LWFPLKDIVKHVEDHCHV | 18 aa @ 237-254 | 1.158 | 100% | 2e-11 | 89% *T. suis* | 3e-09 |

*Selected peptide highlighted

**Our selected peptide section: TECVKPPAHDCPAFG**, 15 aa, from 214 – 228

**Accession number UniProtKB:** A0A0077Z5Q5

**Gene:** TTRE_0000228401

**MW:** 50.494 kDa

**Theoretical pI:** 7.09

**Molecular function:** unknown

**Biological process:** unknown

**Subcellular location:** unknown

**PTM/Processing:** none

**Family & Domains:** unknown

**Pfam model:** unknown

**FASTA sequence:** selected peptide in bold

MVDDGGGTSSDETVPFAWPIVNRTDNDQWSPSTPTQPAPLPLISIPMGYALADHHDHCPA
WKDWRPWTDCLWYPPQNMYTKIAHACGMHAERNLTGVMELPGEAKPPPMCGHCSFKFRCR
RRDNAKDCYPLDGEVDVCHDHGQICTLPKLPHLGCGYAFINEKLKQCFTRPDTPSYVRLG
YRKMFESIPKKHCIEKNGMCKCCCGDYEPNESG**TECVKPPAHDCPAFG**PPGEWSECLWFP
LKDIVKHVEDHCHVHPEADGYRPPVVTPPGIHIPEKCGYCSFRVKCMKRDKKDGCFELKL
GKKSCGHDDCPTCSDICTMDKINGSCVFPRKLKEKLWDDFTAKSKEKHMPHWKRDGYAKL
LMQLPYSNCKEVGGKCKCCCHPYHPNEDGSKCVVKEYCKRVHELHDHDHHHHHHAEHHHS
SSSEDKDHHHKDHAHKEHHHR

**3D structure databases:** created April 15th, 2021.

1.  **SWISS model EXPASY:** P43 (homolog protein from *T. muris*) (Bancroft *et al*., 2019),

    6qix.1.A, identity 83.33% (https://swissmodel.expasy.org).
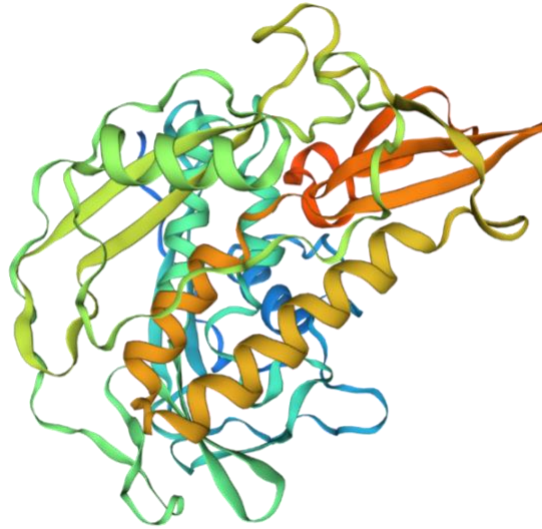


Figure 4.14 3D model Poly-cysteine and histidine tailed protein isoform 2.
*Model based on known protein identities
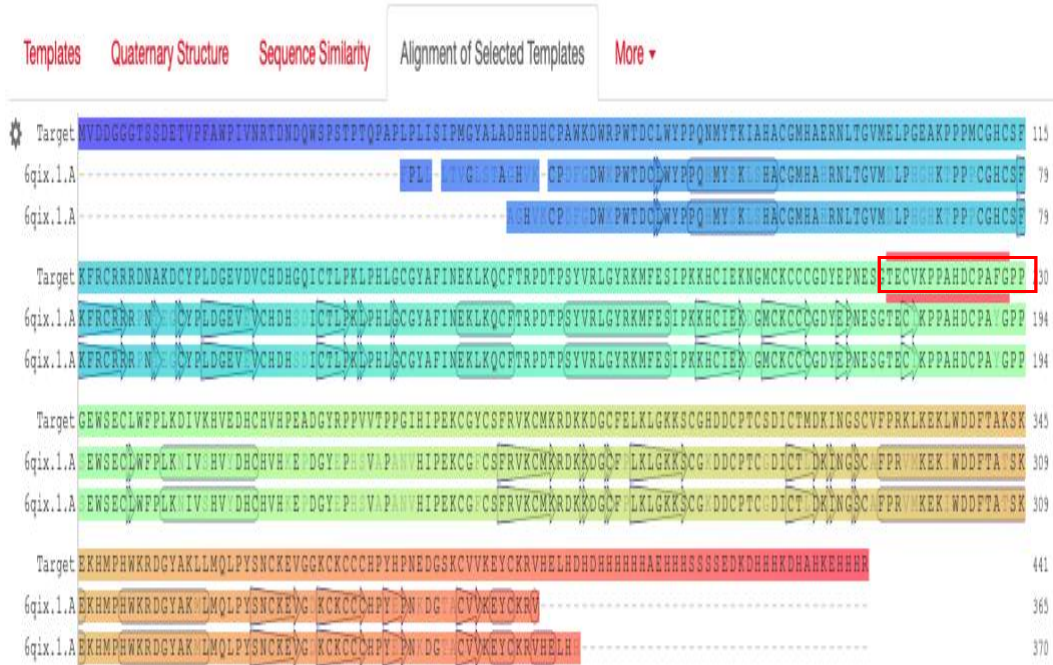
**Figure 4.15 Alignment of known protein identities.**
Selected peptide outlined in red: **TECVKPPAHDCPAFG**, 15 aa, from 214 − 228

### 4.4.2 Synthetic peptides diagnostic feasibility evaluation through Indirect ELISA

The diagnostic feasibility of the selected peptides derived from the four final protein candidates VgNVD (YRSLAIDDVLTETAP), KBDCP (QERCVATLPASVICRLP), CBM14 (SAPTTVTTQLSPVDCVTL), and PCHTP-2 (TECVKPPAHDCPAFG) was evaluated with 19 serum and 19 saliva samples from *T. trichiura* naturally infected AGMs and 6 negative human sample controls. The experiments were performed according to section **3.7.3**. **Figure** 4.16 and **Figure** 4.17 show the indirect ELISA results from each individual saliva sample against each of the four peptides, and **Figure** 4.18 and **Figure** 4.19 show the results from each individual serum sample against each of the four peptides.

In both, the saliva and serum samples, the peptides that elicited the strongest recognition by antibodies were the KBDCP (QERCVATLPASVICRLP) an FE stage specific protein of low abundance, and the VgNVD (YRSLAIDDVLTETAP) an EE stage specific protein.
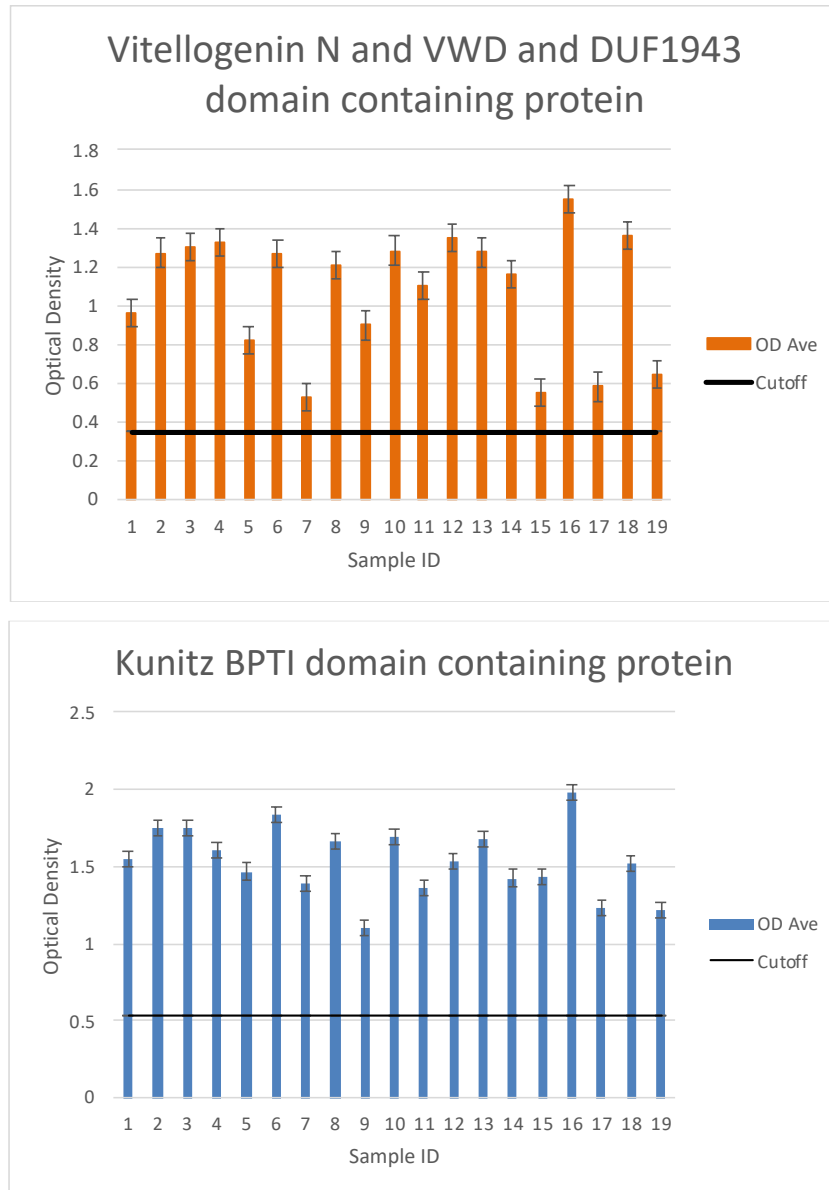
**Figure 4.16** Optical densities from 19 saliva samples from *Trichuris trichiura* infected AGMs analyzed with indirect ELISA against peptides VgNVD (YRSLAIDDVLTETAP), KBDCP (QERCVATLPASVICRLP).
Each saliva sample was analyzed in duplicate. The cutoff was established as the mean OD of the negative controls plus 3 SDs.

The egg protein CBM14 peptide (SAPTTVTTQLSPVDCVTL), and the most abundant protein in all developmental stages of the parasite PCHTP-2 peptide (TECVKPPAHDCPAFG) presented the weakest signals (optical density, OD). Overall, the saliva samples showed strongest optical density values and lower cutoff values than the serum samples.
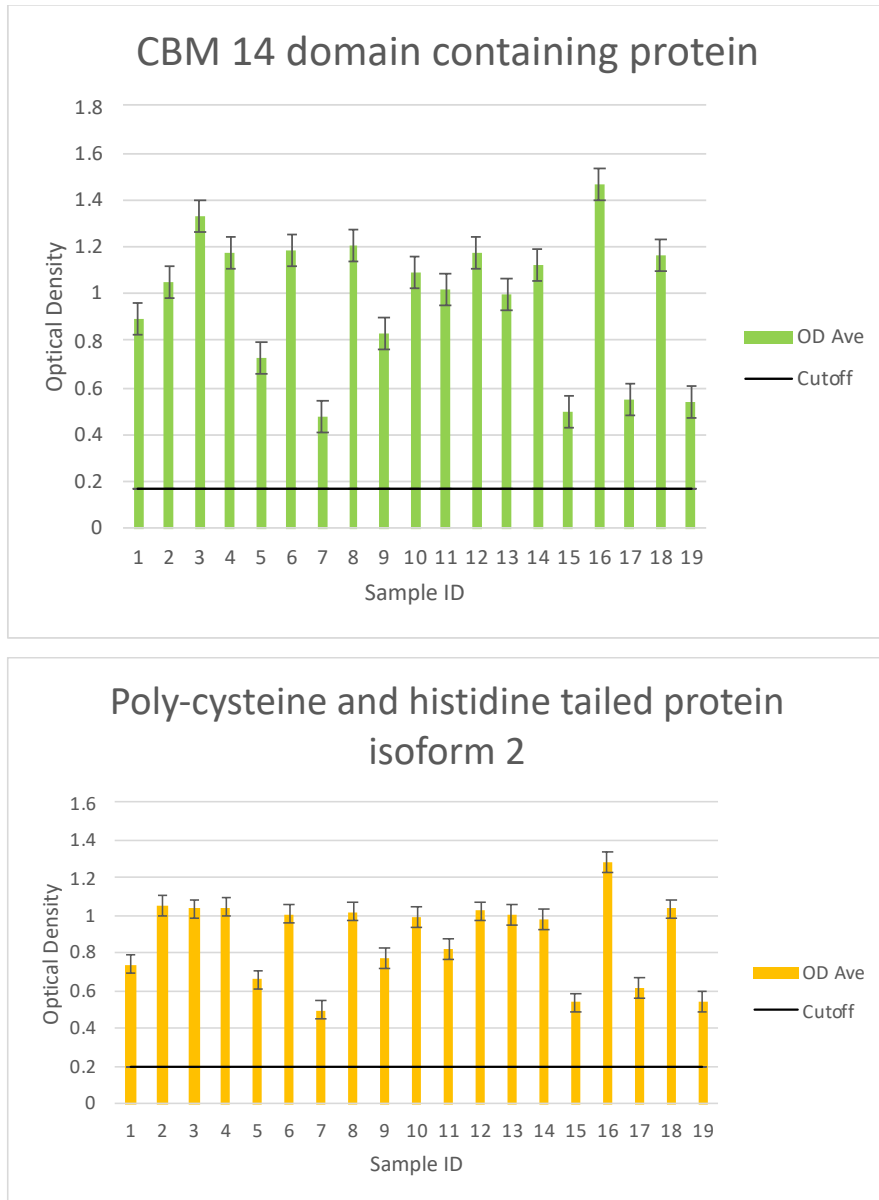
**Figure 4.17 Optical densities from 19 saliva samples from *Trichuris trichiura* infected AGMs analyzed with indirect ELISA against peptides CBM14 (SAPTTVTTQLSPVDCVTL), and PCHTP-2 (TECVKPPAHDCPAFG).** Each saliva sample was analyzed in duplicate. The cutoff was established as the mean OD of the negative controls plus 3 SDs.

The peptides of *T. trichiura* tested by indirect ELISA against 19 *T. trichiura* positive serum samples showed similar results as the saliva samples with an overall weaker antibody recognition.
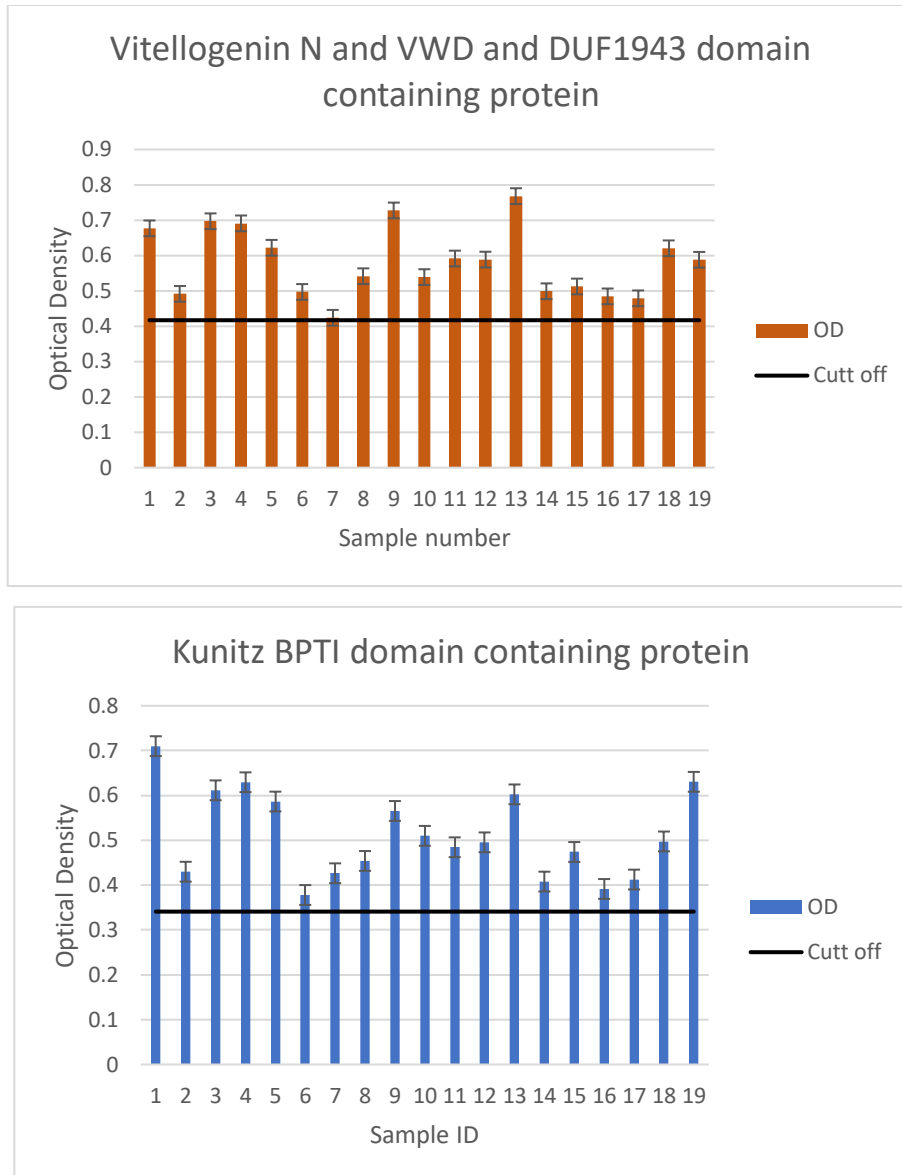
**Figure 4.18 Optical densities from 19 serum samples from *Trichuris trichiura* infected AGMs analyzed with indirect ELISA against peptides VgNVD (YRSLAIDDVLTETAP), KBDCP (QERCVATLPASVICRLP).**
Each serum sample was analyzed in duplicate. The cutoff was established as the mean OD of the negative controls plus 3 SDs.

The egg protein peptide CBM14 (SAPTTVTTQLSPVDCVTL), and the abundant protein PCHTP-2 peptide (TECVKPPAHDCPAFG) candidates presented the overall weakest signals (optical density, OD).
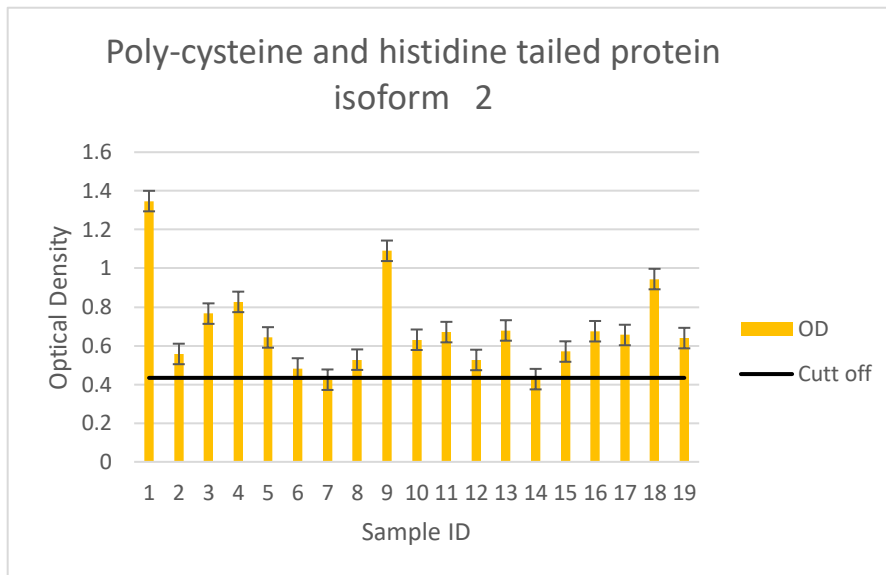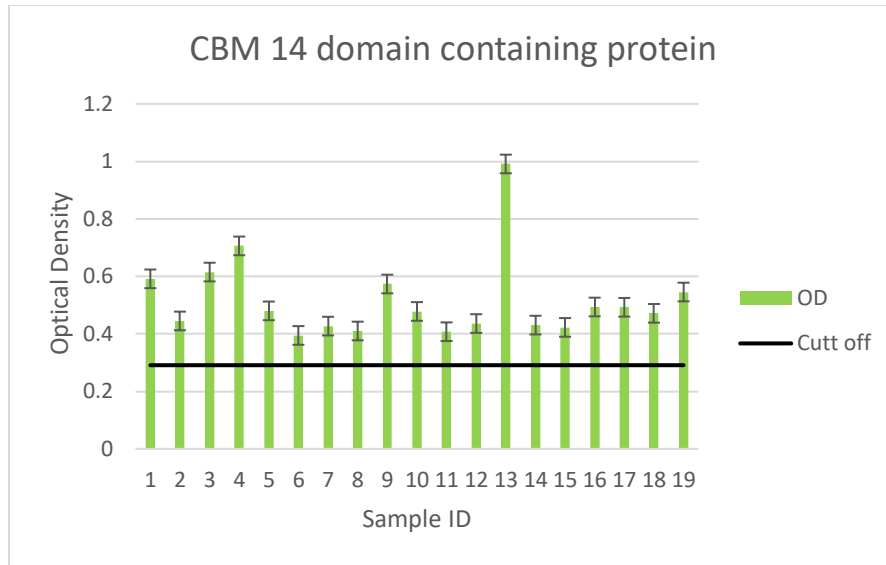
**Figure 4.19 Optical densities from 19 serum samples from *Trichuris trichiura* infected AGMs analyzed with CBM14 (SAPTTVTTQLSPVDCVTL) and PCHTP-2 (TECVKPPAHDCPAFG).**
Each serum sample was analyzed in duplicate. The cutoff was established as the mean OD of the negative controls plus 3 SDs.

Unfortunately, since all positive samples resulted in positive results and all the negative samples resulted in negative results, it was not possible to provide further clarification regarding the specificity and sensitivity analysis.

## 4.5    *Trichuris trichiura* Scanning Electron Microscopy (SEM) images

The head reveals a small slit-like mouth opening with the oral stylet protruding outward (arrow). The stylet appears pointy and slim. The thin anterior portion surrounding the month opening does not display a specific cuticular pattern and immediately after a straited cuticular pattern is observed (**Figure** 4.20, **(A)**). The tick posterior end of the female displays a striated cuticular pattern with transverse grooves that taper at the anal opening (arrow) (**Figure** 4.20, **(B)**).
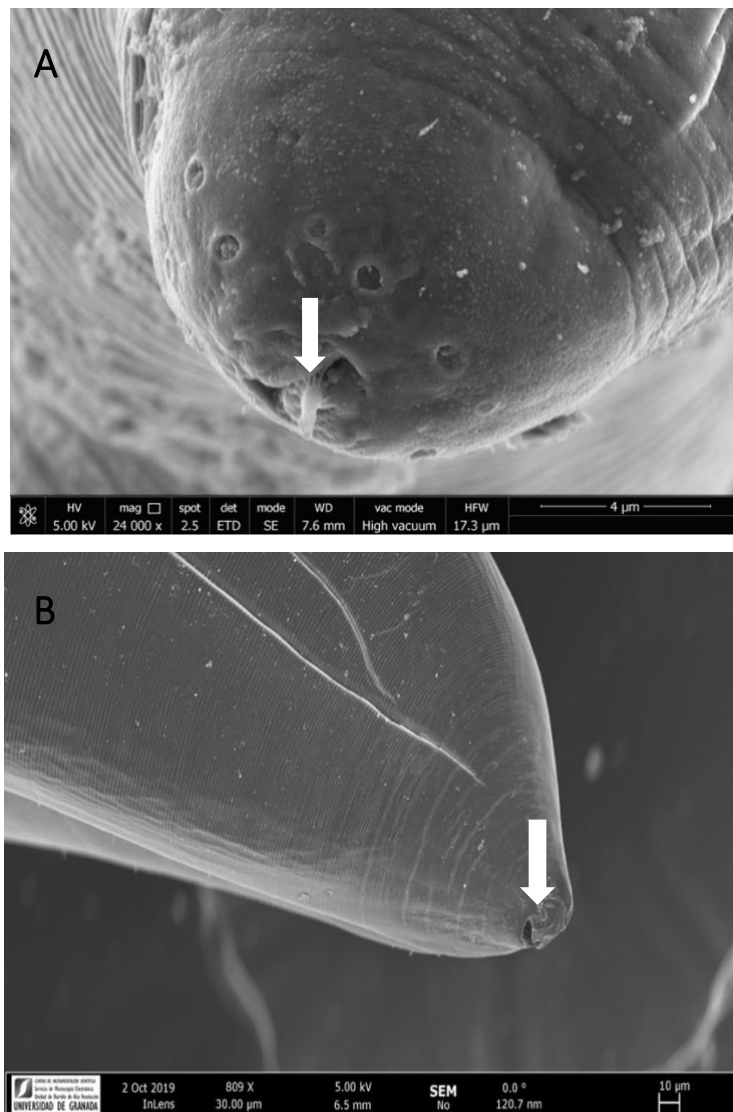


**Figure 4.20 Adult female *Trichuris trichiura* anterior and posterior.**
(A) Adult *Trichuris trichiura* anterior showing stylet (arrow) protruding from mouth opening. (B) Adult *Trichuris trichiura* posterior showing anal opening (arrow).

Figure 4.21(A) and (B) display the difference between a fully developed adult female vulva opening surrounded by an elevated rim-like structure deeply indented with a spinose cuticular appererance and a larval stage, possibly L2, with less defined anatomical structures.

The posterior extremity of males is equipped with a spicule enclosed in a spinose, retractile, cuticular sheat as observed in **Figure** 4.22 (C).
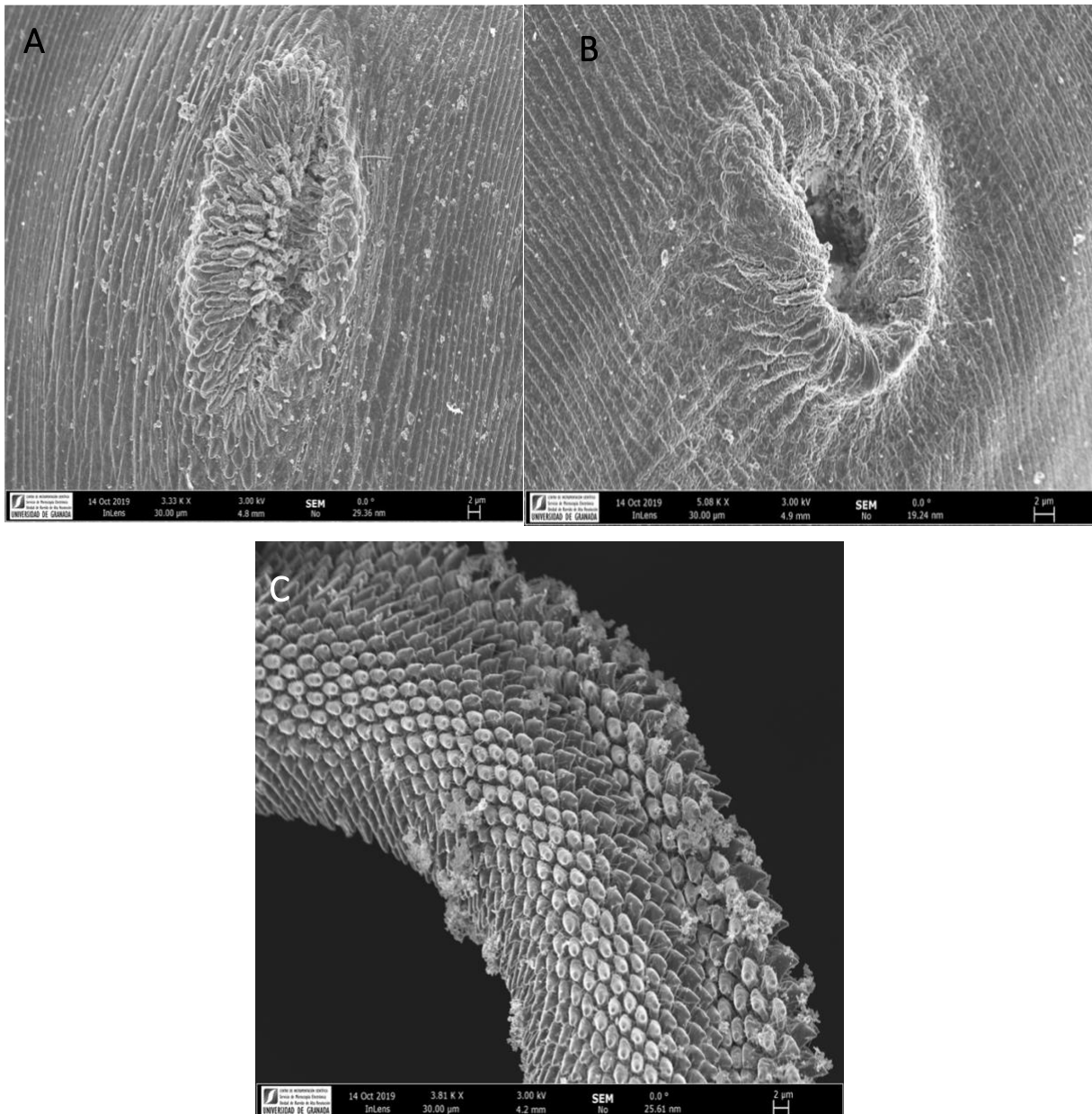


Figure 4.21 Adult *Trichuris trichiura* female and male reproductive structures.
(A) Adult *Trichuris trichiura* female vulva opening. (B) Larval stage *Trichuris trichiura* female vulva opening. (C) Adult *Trichuris trichiura* male spicule structure.

## 4.6    Histopathology

The histopathology analysis from full thickness cecum samples from 33 AGMs did not reveal abnormal morphological or structural changes suggestive of *Trichuris* infection. No other parasites were observed. See **Figure** 4.22.
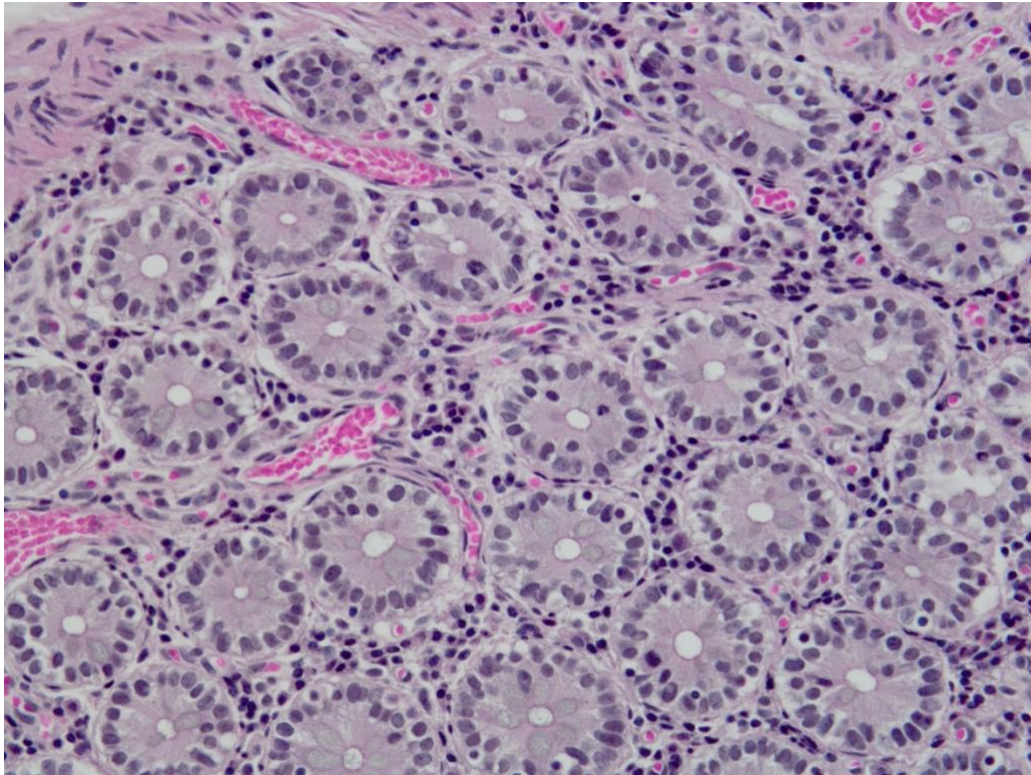


**Figure 4.22 Crypts of Lieberkühn within normal limits.**
The 5 μm AGM cecum sections stained with hematoxylin-and-eosin (H&E) and examined with an Olympus DP73 microscope (40X) (Tokyo, Japan).

# 5 Discussion

## 5.1 Control of nematode infections in captive *Chlorocebus sabaeus*

### 5.1.1 Nematode species present in captive *Chlorocebus sabaeus*

Our study has shown a wide variety of nematodes can be found in captive AGMs on St. Kitts. Many of these have also been described in people on the island and many are regarded as zoonotic. *Trichuris trichiura* was present in 58% of the AGMs and is very common in people worldwide and also on St. Kitts (58% prevalence in 1991; Rawlins *et al.*, 1993). Strongylids species were present in 50% of the AGMs, and hookworm infections were prevalent in St. Kitts in the early 20th century (21.2 %; Annual Reports, Rockefeller Foundation, 1924), but through intensive anthelmintic administration, sanitation, and health education the hookworm disease is no longer a public health problem (Tikasingh *et al.*, 2011). *Strongyloides fuelleborni* was present in 12% of the AGMs and has not been reported in St. Kitts AGMs, further molecular studies are indicated to confirm that the AGMs on the island remain infected with *S. fuelleborni* introduced with the original animals from Africa.

We were unable to determine the *Capillaria* species present, as eggs do not have species-specific morphologies. To date only one species has been described in AGMs. *Capillaria hepatica* (syn. *Carlodium hepaticum*) was found at necropsy in the liver of one animal (*C. aethiops*) in South Africa (*Fripp et al., 1974*). Although *C. hepatica* occurs worldwide, local experience suggests this nematode is not present on St. Kitts as the adult nematodes or associated lesions have never been observed in AGM's liver necropsied on the island, and human hepatic capillariasis has not been reported (Berger, 2020). Similarly, *Capillaria brocheri (*syn. *Aonchotheca brocheri)* (Justine *et al.*, 1988) and *Aonchotheca annulosa* Dujardin, 1845 (nematoda, Capillariidae) (Umur *et al.*, 2012) are the only other species described in NHPs, but they have also not been found in necropsies of AGMs on St. Kitts. Intestinal capillariasis by *Capillaria philippinensis* (syn. *Paracapillaria philippinensis*) has not been described in people from the island (Berger, 2020). Infections, then, might have only been acquired after the animals were introduced onto St. Kitts over 200 years ago.

Whipworms were the most common nematodes found in AGMs in previous reports (Munene *et al.*, 1998; Muriuki *et al.*, 1998; Mutani *et al.*, 2003; Gillespie *et al.,* 2004., Legesse *et*

al., 2004; Petrášová *et al*., 2010; Gaetano *et al*., 2014; Kooriyama *et al*., 2012; Amenu *et al*., 2015; Li *et al*., 2015; Wren *et al*., 2015; Dalimi *et al*., 2016; Valenta *et al*., 2017; Teklemariam *et al*., 2018; Barbosa *et al*., 2020), and were the second most prevalent in our study. *Trichuris trichiura* is the species generally thought to be present in AGMs and this has been confirmed to be the species infecting *C. sabaeus* on St. Kitts (Hawash *et al*., 2016; Yao *et al*., 2018). While most infections in NHPs are subclinical, non-responsive to treatment diarrhea and inappetence, have been reported in fatal *Trichuris* infections with hemorrhagic typhlitis and ileal intussusception found at necropsy (Hennessy *et al*., 1994; Emikpe *et al*., 20002; Eo *et al*., 2018). Recently, chronic whipworm infections have been reported to exacerbate egg-induced hepatopathology in nonhuman primates caused by *Schistosoma mansoni* (Le *et al*., 2020) and have been associated with behavioral shifts (Chapman *et al*., 2016); the affected AGMs had reduced rates of movement and socialization. Infections in people can cause clinical signs including diarrhea, abdominal pain, and in severe cases rectal prolapse (Stephenson *et al*., 2000).

Strongylids such as *Oesophagostomum* spp. *and Trichostrongylus* spp.*,* and the hookworms are the second most common nematodes found in most previous AGMs reports (McGrew *et al*., 1989; Muriuki *et al*., 1998; Mutani *et al*., 2003; Gillespie *et al.,* 2004., Legesse *et al*., 2004; Petrášová *et al*., 2010; Kooriyama *et al*., 2012; Amenu *et al*., 2015; Wren *et al*., 2015; Dalimi *et al*., 2016; Valenta *et al*., 2017; Teklemariam *et al*., 2018; Blersch *et al*., 2019). Although they were the third most prevalent in our study they were not reported in previous studies on St. Kitts (Cameron, 1930; Ritchie *et al*., 1967; Hawash *et al*., 2016; Yao *et al*., 2018, Gallagher *et al*. 2019). The morphology of the different strongylid eggs does not enable species-level identification; however, we were able to use egg morphology to re-examine the samples and confirm that hookworms (Ancylostomatiodea) and *Trichostrongylus* spp. were present in all AGMs (Hasegawa, 2009; Modrý *et al*., 2015; Blersch *et al*., 2019).

Adult hookworms reside in the small intestine and *Trichostrongylus* spp. in the stomach and small intestine, but molecular analysis is needed to determine the species. Hookworms (*Necator* spp. or *Ancylostoma* spp*.*) are zoonotic (Hasegawa *et al*., 2014) and can cause severe anemia. *Trichostrongylus* occurs primarily in ruminants (Acha *et al*., 2003) and strongylid species are routinely detected in ruminants on St. Kitts, which has large populations of free-roaming

sheep, goats, and cattle. Humans can be infected, albeit infrequently, and AGMs are likely infected when exposed to larvae while foraging on lands where ruminants have grazed. Infections in NHPs are mostly asymptomatic, but in people with acute trichostrongyliasis there can be transitory eosinophilia and digestive disorders (Acha *et al*., 2003).

*Strongyloides* spp. have been reported in AGMs from St. Kitts (Ritchie *et al*., 1967; Gallagher *et al*., 2019) but, although they are the third most common nematode reported in previous AGMs studies (McGrew *et al*., 1989; Munene *et al*., 1998; Muriuki *et al*., 1998; Mutani *et al*., 2003; Gillespie *et al.,* 2004; Petrášová *et al*., 2010; Gaetano *et al*., 2014; Kooriyama *et al*., 2012; Amenu *et al*., 2015; Li *et al*., 2015; Wren *et al*., 2015; Dalimi *et al*., 2016; Valenta *et al*., 2017 and Teklemariam *et al*., 2018), they were the least prevalent in our study. Nonhuman primates can be infected with the major zoonotic species of the genus, *S. stercoralis* and *S. fuelleborni* (Kouassi *et al*., 2015), but we only identified the latter, which is found mainly in tropical regions of Africa and Asia (White *et al*., 2019). Mild infections with *S. fuelleborni* are mostly asymptomatic and self-limiting with occasional diarrhea in immunocompetent animals, but massive infections in young or immunocompromised animals can cause bronchopneumonia, pulmonary hemorrhage, intense diarrhea, anorexia, emaciation and reduce growth (Acha *et al*., 2003; Cogswell, 2007).

While two different genera of strongylid species were identified in our AGMs, further molecular studies are warranted to confirm if AGMs are hosts to more than one strongylid species and to determine the species of *Capillaria*.

### 5.1.2  Treatment and control outcomes

Our data further emphasize the importance of establishing the most effective treatment regimen for captive monkeys to prevent zoonotic nematodes from spreading to animal handlers and other people that might come into contact with AGMs feces and contaminated soil. Establishing the most effective and least invasive treatments will help in ensuring optimal health and minimal stress in captive monkey populations.

Our findings that captive *C. sabaeus* on St. Kitts had high infection rates with a variety of nematodes post-PRAL treatment regimen indicates this is not an effective treatment regimen.

Praziquantel is mainly effective against cestodes and trematodes (Utzinger and Keiser 2004; Geary *et al*., 2010). Although St. Kitts was endemic for human schistosomiasis in the 20th century, with AGMs also infected with *S. mansoni* (Cameron, 1928), the disease was eliminated from the island in the 1960s (Hewitt and Willingham, 2019) and cestode and trematode infections are now very rare on the island (Berger, 2020). Therefore, the use of praziquantel on the AGMs of St. Kitts appears unnecessary unless guided by a specific diagnosis. On the contrary, benzimidazoles such as albendazole, are used for the control of helminthiasis such as *Ascaris lumbricoides*, *T. trichiura,* and hookworms (Geary *et al*., 2010) and also for *Capillaria* sp. (Cross, 1992). As three of these nematodes were found in our AGMs, (*Capillaria*, *Trichuris*, strongylids) the drug appears indicated for treatment.

Although albendazole has ovicidal and larvicidal activity by binding to intercellular tubulin (Dayan *et al*., 2003), repeated consecutive doses of the drug are needed to achieve optimal efficacy in people (Geary *et al*., 2010) and in AGMs (Kagira *et al*., 2011). The PRAL treatment regimen based on existing recommendations (Junge, 2015; nonhuman primate formulary, 2018) that we used consisted of two doses of albendazole, but these were not consecutive which most likely explains the poor results of the regimen. The use of three consecutive doses of albendazole in the IVAL treatment, however, proved better efficacy with 64% of the *C. sabaeus* having a negative FEC for all parasites three months post-treatment; the four animals remaining positive by FEC had only *T. trichiura*.

Macrocyclic lactones such as ivermectin are potent and safe against adult and larval nematodes by inhibiting their motion and feeding (Martin *et al*., 1997). The disadvantage is that when only a single dose is given, the inhibition can be temporary and may only reduce the fecundity of the female decreasing the egg output (Kagira *et al*., 2011; Wang *et al*., 2008), instead of triggering their death or expulsion. This can potentially lead to the recovering parasites continuing to produce eggs (Petersen *et al*., 1996), a situation that can result in false negative results if the post-treatment monitoring times are short and fall below the parasite prepatent period (Reichard *et al*., 2017). The three consecutive daily doses in our IVAL treatment protocol would seem to prevent the above problem and have resulted in more effective expulsion of nematodes. Although it has been shown there is no potentiation synergism between ivermectin

and albendazole (Entrocasso *et al.*, 2008), when used together, the drugs have shown to have better efficacy against nematodes (Olsen, 2007; Geary *et al.*, 2010).

Although we generally observed good results with the IVAL regimen there were apparent treatment failures that warrant further comment. Four AGMs had *T. trichiura* eggs three months after one treatment with IVAL but became negative after a second treatment. Although there are many possible explanations, we think it most likely the IVAL treatment was carried out during the prepatent period of *T. trichiura*, which might be as long as 16 weeks (Hansen *et al.*, 2016). It is also possible the animals became infected while in captivity, which is the most likely explanation for the one AGM that was negative for *T. trichiura* before, and 3 months after, the first IVAL treatment but was positive for adults and eggs at necropsy 8 months later.

*T. trichiura* eggs can remain inactive on surfaces for years (Bundy and Cooper, 1989) and have been found on fruits and vegetables (Kłapeć *et al.*, 2012). Although the AGMs cages in our study were cleaned and disinfected regularly, eggs are difficult to inactivate (Bundy and Cooper, 1989). It is of note that six of these seven animals that were negative by FEC three months after the one IVAL treatment were still negative six to eight months post-treatment. This indicates the hygiene measures in place were mostly effective at preventing reinfection, which is difficult as *T. trichiura* eggs can survive temperatures of 50 ˚C and − 9 ˚C for 5 days and 100% humidity for a week (Bundy and Cooper, 1989). They also resist short wave radiation (180 − 315 nm) (Nolf, 1932) and the eggshell only dissolves in 10% sodium hypochlorite after 25 hours of immersion (Wharton and Jenkins, 1978). Of the husbandry practices used in our study, it would appear that the steam cleaning (>82 ˚C) could yield better control of reinfection.

The fruits and vegetables fed to our AGMs may have been a source of reinfection as they were only washed if evidently soiled. However, it is known that nematode eggs can be present on fruits and vegetables even after excess soil is removed (Kłapeć *et al.*, 2019). Although water has been shown to be a source of infection with a variety of nematode eggs (Noda *et al.*, 2009), the smallest nematode eggs found in our study are larger (20-30 μm wide) than the 5 μm filters used in the water supply for our study animals. Although the water was also treated with UV light, a recent meta-analysis concluded that this is not very efficient for controlling parasites (Hazell *et al.*, 2019). Finally, cockroaches and flies can be mechanical transmitters of nematode eggs (El-Sherbini

and El-Sherbini, 2011) a challenge to control fully in the partially open environment at the SKBRF facilities.

All of the eight AGMs that were positive for *Capillaria* sp. eggs before IVAL treatment were negative at 3 months post-treatment. However, four AGMs (50%) subsequently became positive, all despite a second IVAL treatment. As the morphology of *Capillaria* sp. eggs cannot be used for speciation and no *Capillaria* were reported in the necropsy findings we could not determine the specie/s infecting our AGMs. Without knowledge of the infecting specie/s life cycle/s it is not possible to determine if these AGMs that became positive had been in the prepatent period during treatment, were autoinfected, reinfected, or had false-negative test results previously.

The presence of single-sex infections and the lack of correlation between egg output and total nematode or female counts at necropsy highlights that fecal egg counts have an overall low diagnostic sensitivity. Inaccurate estimations can be obtained when worm loads are calculated based on egg counts (Kannangara, 1975), contributing to the lack of adequate and active diagnosis of infections.
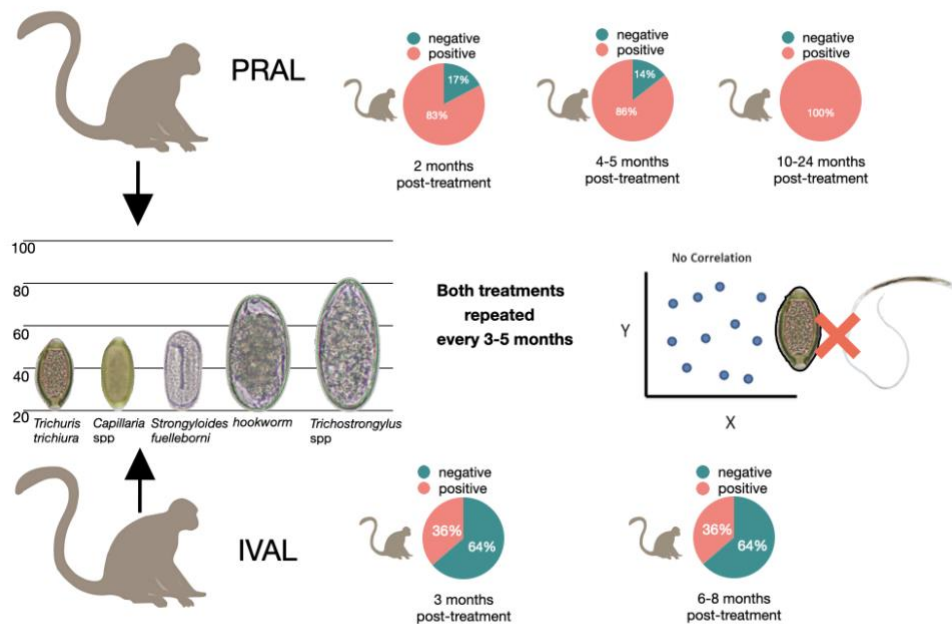


Figure 5.1 Graphical representation of treatment results evaluated in captive AGM

## 5.2  *Trichuris trichiura* immunogenic proteins of diagnostic value

### 5.2.1  Diagnostic challenges to overcome

Recent reports confirm that most cases of *T. trichiura* infections remain undiagnosed (Jourdan *et al.*, 2018), and chronic infections can remain undetected for years (Else *et al.*, 2020). This is because diagnosis of infections is based on the detection of eggs through coprological analyses that do not predict true parasite loads or real-time infection status. Early diagnosis of trichuriasis and diagnostic methods that do not rely on inconsistent clinical signs or fecal analysis are crucial to detect the infections following accidental ingestion and during the prolonged prepatent period of the parasite. At present, there are only limited data on *T. trichiura* antigens that can be used in serological diagnostic tests and the purpose of this study is to present the first description of the *T. trichiura* egg proteome and the immunodominant proteins present in both EE and FE. We also consider that the method described for *T. trichiura* egg isolation would be suitable for isolating large amounts of eggs from a more sterile and practical environment than the feces and, although the in-uteri eggs are not yet embryonated, they do present somatic and excretory/secretory proteins of the eggshell and those of dividing embryonic cells. We are demonstrating that their immunoproteomic analysis provides valuable information that warrants further study.

### 5.2.2  *Trichuris trichiura* genome and other helminths provide insights into the egg proteome.

In *T. trichiura*, the parasite-host interactions are poorly understood and are highly influenced by the parasite's life cycle. Limited information on stage-specific antigens, immune evasion strategies and immunomodulatory effects have been described in animal models of *T. muris* and *T. suis* (Leroux *et al.*, 2018; Shears *et al.*, 2018; Bancroft *et al.* 2019). Foth and collaborators (Foth *et al.*, 2014) described the whole-genome of the human-infective adult *T. trichiura* and we can now compare the *T. trichiura* egg proteome to their findings; they also identified numerous genes that are differentially expressed in a sex- or stage-specific manner. The most abundant transcripts found in this extensive study included proteins we have now definitively identified in the EE proteome, such as two WAP domain containing SLPI-like proteins, protease

inhibitors such as cystatin-domain containing protein and nematode cuticle collagen N-terminal domain containing proteins and chitin binding domain containing proteins such as CBM14 domain containing proteins.

Furthermore, with more or less representation, but of particular interest within the context of the present work, we have found *Trichuris* egg proteins with known immunomodulatory properties such as macrophage migration inhibitory factor homolog (MIF), previously identified in *T. trichiura* adult (Santos *et al*., 2013), and 14-3-3 protein which has also been identified in several developmental stages of other nematodes, *Trichinella britovi* (Grzelak *et al*., 2018) and *Trichinella spiralis* (Yang *et al*., 2016) and trematodes, *Schistosoma japonicum* (Luo *et al*., 2009). Both proteins are considered as enhancers of humoral and cellular immune responses (da Silva *et al*., 2018). Although their function and biological process in *T. trichiura* remains unknown we are confirming the presence in the EE proteome and highlighting their potential role in the initial stages of the parasite-host interaction.

### 5.2.2.1 EE proteome proteins with the largest numbers of distinct peptides

*Lipid transporter and major secreted protein with unknown function*. Interestingly, two of the proteins identified with the largest numbers of distinct peptides in the EE proteome presented in our work, VgNVD and PCHTP-2, were also among the top 25 most abundant transcripts found by Foth and collaborators (Foth *et al*., 2014). Vitellogenins are a lipid transfer proteins, they play a significant role in embryonic development and are extensively conserved amongst nematodes (Chen *et al*., 1997). They provide the growing embryo with amino acids (Vercauteren *et al*., 2003), therefore VgNVD being the most abundant protein in the EE proteome represents an important antigenic target that can be consistently identified in eggs and adult females. The detection of PCHTP-2 in the EE proteome as the second most frequently detected protein is in accordance with Shears and collaborators (2018) who found it to be the most abundant protein in the *T. muris* adult secretome. Even though a specific function has not been assigned yet for *T. trichiura*, Bancroft and collaborators (2019) identified PCHTP-2 as the most abundant protein in cecal mucus from chronically infected mice with *T. muris* and confirmed its expression in all developmental stages

confirming PCHTP-2 as the major secreted protein of the whipworm despite not presenting signal peptide.

*Energy and metabolism.* One of the most represented groups of proteins we found in the egg proteome were those related to energy and metabolism and included proteins associated with glycolysis (enolase and glyceraldehyde-3-phosphate dehydrogenase (GADPH), gluconeogenesis (triosephosphate isomerase and phosphoenolpyruvate carboxykinase GTP) and other metabolic enzymes such as alpha-1,4 glucan phosphorylase and malic enzyme. This fact is consistent with previous studies in which these metabolic enzymes were described on the surface of the helminths, nematodes, and trematodes, found to participate in oxidative processes, parasite invasion and migration processes within the host (Sotillo *et al.*, 2008; Bernal *et al.*, 2006; Dea-Ayuela *et al.*, 2005; Pérez-Sanchez *et al.*, 2008; Sotillo *et al.*, 2011; Sotillo et al., 2012).

*Muscle, motility, and cytoskeleton.* The ensuing functional group with the largest number of representatives was related to the cytoskeleton, muscle, and motility. Actin, tropomyosin, paramyosin, intermediate filament protein IFA 1 and epididymal secretory protein E1 were found with a high number of distinct peptides. These proteins are essential to enhance the motility of nematodes and have also been recorded in many helminthic proteomes: somatic extract of adults of *T. spiralis* (Yang et al., 2015), *T. britovi* (Grzelak et al., 2018), *Syphacia muris* (Sotillo et al., 2012), and *Echinostoma caproni* (Sotillo et al., 2010); and in egg secretions of *Schistosoma mansoni* (Cass et al., 2007). Specifically, intermediate filament protein IFA1 has been studied in *Caenorhabditis elegans*, demonstrating that in nematodes and potentially similar for *T. trichiura*, they allow epidermal elongation in the larval stages to grow into adults (Woo et al., 2004).

*Survival: antioxidants and chaperones.* We also found proteins essential for the survival of the nematode within its host, in the hostile conditions of the cecum, during stress and for detoxifying processes including antioxidants and chaperones. The Cu/Zn superoxide dismutase (Cu/Zn-SOD) was found in the EE proteome and has also been identified on *Trichuris ovis* homogenates (Sanchez-Moreno *et al.*, 1992), the adult surface and larval extracts (secreted and somatic) of *Toxocara canis* (da Silva *et al.*, 2018), in the somatic extract of adults of *Fasciola hepatica,* and the *S. mansoni* egg secretome (Cass *et al.*, 2007; Kim *et al.*, 2000). This essential

enzyme antagonizes the host's inflammatory responses by regulating the free radical balance and reactive oxygen species in cells protecting helminths against cell death (Cardoso *et al.*, 2004). Heat shock proteins (HSP90, HSP70, HSP60) are inducible conserved proteins widely described in parasite proteomes and secretomes, and we have confirmed their presence in the EE proteome. They act as molecular chaperones which fold, assemble and translocate other proteins to ensure the survival of the parasite by defending it against stressful situations being important in stress tolerance (Higón *et al.*, 2008). Small heat shock proteins HSP20 and HSP20 domain containing protein were also identified in EE proteome, which are known to aid parasite survival under hostile conditions such as heat or nutritional stress (Pérez-Morales *et al.*, 2015).

*Signaling.* Within the proteins implicated in signaling pathways, we identified galectin in the EE proteome, a type of lectin found in different extracts of nematodes such as adults and larvae of *T. canis* (da Silva *et al.*, 2018) and extract of infective larvae (L3) of *Haemonchus contortus* (Turner *et al.*, 2008) with a role in immune signaling pathways. Nematode galectins are believed to be immunological mediators with implications in survival and interaction with the host (Young *et al.*, 2002) and modulate a range of immune responses, including the cellular immune response, inflammatory processes, and immune regulation (Shi *et al.*, 2018) all essential for prolonged survival of *T. trichiura* in the host.

## 5.2.2.2 Antigenic profile of *T. trichiura* EE and FE extracts and identification of the top immunodominant proteins

Previous studies have used an immunoproteomic approach to determine the antigenic proteins of helminths at different developmental stages (larvae and adults) (International Helminth Genomes Consortium, 2018) and the serological responses to soluble protein extracts of *Ascaris lumbricoides* (Acevedo *et al.*, 2013), *Trichinella britovi* (Grzelak *et al.*, 2018), *Schistosoma japonicum* (Wang *et al.*, 2013) and *Taenia solium* (Santiváñez *et al.*, 2010).

Parasitic worms, like *T. trichiura* have a remarkable ability to modulate the host immune response through several mechanisms; specific parasite-derived proteins can modulate immune

functions playing an essential role in the parasite-host interaction (International Helminth Genomes Consortium, 2018). Excretion/secretion proteins from larvae and adults of the porcine whipworm, *T. suis*, closely related to the human *T. trichiura*, were investigated by Leroux et al. (Leroux *et al*. 2018), who identified a subset of proteins that promote specific anti-inflammatory functions and immunomodulatory properties. Here we present the combination of proteomic techniques, such as one dimensional gel electrophoresis and tandem mass spectrometry as a comprehensive approach to identify *T. trichiura* proteins of immunodiagnostic value.

**Vitellogenin N and VWD and DUF1943 domain containing protein.** Our findings of VgNVD being a major protein in the EE proteome and having immunogenic value in our naturally infected monkeys is significant as Shears and collaborators (Shears *et al.*, 2018) identified VgNVD in extracellular vesicles (EVs) of *T. muris* as a potential immunogenic candidate. Antigenic homologs of VgNVD have been identified in free-living nematodes such as *C. elegans*, and adult parasites secretomes of *Ascaris suum*, *Nippostrongylus brasiliensis*, *Heligmosomoides polygyrus* and *Litomosoides sigmodontis* (Mei *et al.*, 1997; Moreno *et al.*, 2011; Armstrong *et al*. 2014; Chehayeb *et al.*, 2014; Sotillo *et al.*, 2014; Ibáñez-Shimabukuro et al., 2019) and also in *H. polygyrus* eggs (Hewitson *et al.*, 2013) which confirms the significance of our results in the context of current efforts to identify potential diagnostic, vaccine or drug targets. The VgNVD was not a distinct immune complex of interest identified in the FE alone when compared to other nematode banding patterns, therefore its presence in the FE was not identified.

**Heat shock protein 70.** HSP70 and heat shock proteins, in general, have caught the attention of researchers for acting typically as immunodominant antigens eliciting strong humoral responses as major targets of host immune responses, suggesting them out as possible candidates for antiparasitic, allergic and autoimmune diseases treatments (Mayer *et al.*, 2005; Mansilla *et al.*, 2014). Our findings that HSP70 is present in the EE in low abundance is in contrast to other work where the HSP70 is amongst the most highly abundant protein identified in egg secretions of *S. mansoni* and *H. polygyrus* (Cass *et al.*, 2007; Hewitson *et al.*, 2013)17,62]. HSP70 is also heavily represented in *E. caproni, F. hepatica, H. polygyrus, Schistosoma bovis, T. trichiura, T. britovi,* and *Zygocotyle lunata* adult worms extract (Santos *et al.*, 2013; Grzelak *et al.*, 2018); Sotillo *et al.*, 2011;

Sotillo *et al*., 2010; De la Torre-Escudero *et al*., 2011; Hewitson *et al*., 2011) which highlights that for the EE this can be a less abundant target. However, the contrasting finding of low prevalence may be due to the NE stage of the *Trichuris* eggs use in the study. Further studies are warranted to stablish this comparison. Others have reported on their immunogenicity linked to stimulation of IgG and IgM responses (Dea-Ayuela *et al*., 2005; Schmitt *et al*., 2007; Tsan *et al*., 2009), and they have been suggested as possible vaccine targets (Wang *et al*., 2009).

Poly-cysteine and histidine tailed protein isoform 2. PCHTP-2 was the second most abundant in the EE and also present in the FE. This protein was identified as a strong immunogen of *Trichinella pseudospiralis* adult secretome (Wang *et al*., 2017). Another protein of the same family, poly-cysteine and histidine-tailed metalloprotein, implicated in metal storage and/or transport, was the first member of the nematode poly-cysteine protein family described in *T. spiralis* (Radoslavov *et al*., 2010). Since these proteins are unique for parasites of the Superfamily Trichinelloidea, their potential applications in diagnostics and treatment could be exploited in the future (Radoslavov *et al*., 2010) and we show here that in the case of *T. trichiura* PCHTP-2 has a strong presence. Recent work by Bancroft (Bancroft *et al*., 2019) hypothesized that the unique structural features of the homolog protein allows binding to IL-13, which is considered the key effector cytokine responsible for *T. muris* expulsion, able to inhibit IL-13 function both *in vitro* and *in vivo*. Our finding that PCHTP-2 is equally abundant in EE and FE as well as a strong immunogen in our naturally infected AGMs is significant as we can confirm that this protein has a strong presence in both life cycle stages and in accordance with Bancroft (Bancroft *et al*., 2019) in the *T. muris* model. Our results are in agreement with presenting PCHTP-2 as a *Trichuris*-derived immunomodulatory molecule that could serve as a key target for the development of immunodiagnostics, vaccination or drug based therapeutics.

Enolase and glyceraldehyde-3-phosphate dehydrogenase. We identified certain glycolytic enzymes such as enolase and GAPDH, as immunoactive components of the *T. trichiura* EE and FE. Both of them are present on the surface of helminths interacting with the host surface as is the case of the delicate interaction between *T. trichiura* and the enteric cells of the cecum. Furthermore, enolase plays a vital role in the degradation of the intracellular matrix through the

activation of plasminogen facilitating the invasion, migration, and fixation in the host (Foth *et al.*, 2014; Cass *et al.*, 2007; Bernal *et al.*, 2006; Sotillo *et al.*, 2012) all essential mechanisms to ensure *T. trichiura* prolonged survival. In *T. spiralis* (Dea-Ayuela *et al.*, 2005) and *T. britovi* (Grzelak et al., 2018), this enzyme has been confirmed as immunodominant, suggesting that it may assist in tissue migration of the larvae a critical task that *T. trichiura* must accomplish shortly after the hatching from the egg. Enolase and heat shock proteins have also been classified as exosome markers (Shears *et al.*, 2018; Buck *et al.*, 2014) in accordance with our findings of enolase lacking signal peptide. Likewise, GAPDH has been previously linked to fibronectin, laminin, entactin, and collagen binding (Gozalbo *et al.*, 1998) and Cass and collaborators (Cass *et al.*, 2007) suggested that in the case of *S. mansoni* this protein could be involved in the attachment of the eggs to host tissues or aid the passage of live eggs across host tissues to the external environment. Our results suggest GAPDH as having a relative abundance in both EE and FE that could align with Cass (Cass *et al.*, 2007) findings and warrant further study.

**Kunitz BPTI domain containing protein.** With lower abundance but strong immunogenicity presence in the FE, we identified KBDCP, a protease inhibitor protein part of the WAP domain-containing proteins strongly expressed in the anterior region of adult whipworms and likely secreted based on the presence of signal peptide (Foth *et al.*, 2014). Protease inhibitors are involved in protecting the nematodes from host proteinases; they modulate their activity and protect them from degradation. Also, they assist parasite survival by facilitating feeding and manipulating the host response to the parasite (Knox, 2007). Kunitz BPTI domain containing proteins are also the most abundant protease inhibitors across all nematodes species (International Helminth Genomes Consortium, 2018)

**Carbohydrate-binding module 14 domain containing protein.** Present only in the EE, we identified CBM14, a chitin binding domain protein associated with the formation of the eggshells or a related function due to the characteristic presence of chitin in the trichuris eggshell (Wharton and Jenkins, 1978; Foth *et al.*, 2014). Eggshell chitin synthesis starts at fertilization (Johnston *et al.*, 2010) and transcripts for proteins with chitin-binding domains have been described as upregulated in the female whipworm (Foth *et al.*, 2014) confirming the relevance of this finding.

### 5.2.2.3 Diagnostic applicability of the synthetic peptides designed from FE and EE

The advantage in utilizing the smaller amino acid sequences chosen for the design of the synthetic peptides is that these sequence regions can be carefully selected and are specific for *T. trichiura* with a predicted antigenicity. In contrast to utilizing whole proteins of larger size, the possibility of having crossed reactions is more significant due to the presence of common epitopes amongst them. In recent years, there exists a higher tendency to use synthetic peptides derived from key proteins to diagnose other parasitic diseases (List *et al*., 2010; Intapan *et al*., 2013).

Furthermore, the development of serological diagnosis is hampered by the limited availability of antigen. The antigen extracts need to be prepared from live worms. Access to worms from humans is scarce, and limited populations of NHP offer the possibility of harvesting parasites as an alternative source of parasitic material. Here we show the advantage of using the captive wild-caught population of the St. Kitts Biomedical Research Foundation & Virscio as disease and disease-free animal model. Varying methods of antigen preparation makes inter-laboratory standardization difficult. Therefore, the use of synthetic peptides as a diagnostic antigen is a good alternative. The peptide ELISA for detecting of IgG4-specific antibodies improved the diagnostic value for neurocysticercosis (Intapan *et al.,* 2008) and fascioliasis (Intapan *et al*., 2005; Tantrawatpan *et al*., 2007). Also, for filariasis (Pandiaraja *et al*., 2010), cestodes such as *Echinococcus granulosus* (González-Sapienza *et al*., 2000) and trematodes such as *S. mansoni* (Noya *et al*., 2003; Oliveira *et al*., 2006). Immunodiagnostic methods have expanded with time and now still remain one of the most robust tools for diagnostic of parasitic diseases.

Based on the sequences of our final four protein candidates characterized in our laboratory with the best immunogenic potential using saliva and serum from naturally infected nonhuman primates, four peptides were synthesized. The amino acid regions selected had no homology with other nematodes. To evaluate their diagnostic capacity, the indirect ELISA assays were performed with serum and saliva from naturally infected nonhuman primates and negative human controls. All the naturally infected positive samples were positive, and all the negative human samples were negative with all four peptide candidates. Therefore, it was not possible to infer further results regarding the sensitivity or the specificity data. Kunitz BPTI domain containing protein and VgNVD

showed the highest reactivity, while PCHTP-2 and CBM14 showed the lowest reactivity with saliva and serum. Saliva samples results, however, were more consistent across all peptides.

Efforts were made to acquire true negative samples from nonhuman primates after treatment, confirmed at necropsy and by histology of the cecum due to the prevalence of trichuriasis being so high on the island. Unfortunately, the positive biological samples had lost their diagnostic value when the disease-free biological samples were identified. Future studies with human positive and negative samples as well as nonhuman primate positive and true negative samples are warranted to further study the diagnostic potential of the four novel diagnostic peptides for *T. trichiura* identified in our work.

# 6  Conclusions

1. A wide range of nematode parasites can be found in captive AGMs on St. Kitts and effective anthelmintic protocols are needed to ensure these animals remain healthy in captivity.

2. All the nematodes identified in the AGMs on St. Kitts are potentially zoonotic and thus AGMs represent a threat to the health of people on the island.

3. In the absence of significant cestode and trematode infections, the IVAL treatment protocol would appear to be effective in controlling nematode infections in captive AGMs as long as sound husbandry practices are in place. In particular, thorough washing of fruits and vegetables, and insect pest control which all help prevent reinfections that can impact optimal health and behavior of study animals.

4. It is important to note that regular FECs will not detect all *T. trichiura* infections. There is no correlation between egg counts and total nematode or female counts at necropsy; therefore, full advantage should be taken of necropsied animals for parasite detection and surveillance.

5. The *T. trichiura* life cycle inside the host starts with the egg hatching and the release of the larva. This period of time remains as an undiagnosed stage. The proteins described in this doctoral thesis are directly exposed to the immune system during this undiagnosed time and, as we demonstrate, can elicit anti-*Trichuris* antibodies by the host, which may have diagnosis applicability.

6. Our study is the first effort to identify the proteome of the NE *T. trichiura* eggs as a novel source of potential diagnostic targets and provides details which might serve for improved diagnostics, identification of immunomodulators, and facilitate treatment and control of this neglected disease.

7. As the infective developmental stage of the nematode, the eggs signal the host interface with their shell surface antigens and the subsequent release of larvae and associated fluids as the first stimuli to the host's immune system. Later in infections, the NE eggs released by the females into the cecum and their secretomes would also be expected to stimulate the hosts' immune system. The NE egg proteome revealed common families of proteins which are known to play roles in energy and metabolism; cytoskeleton, muscle, and

motility; proteolysis; signaling; the stress response and detoxification; transcription and translation; and lipid binding and transport.

8.  This initial list of *T. trichiura* egg proteins (proteome and antigenic profile) can be used in future research into the immunobiology and pathogenesis of human trichuriasis and the treatment of human intestinal immune-related diseases.

9.  The designed synthetic peptides and confirmatory indirect ELISA results are promising and showed that the novel amino acid sequences chosen represent important candidates for future assay development and refinement with either positive human samples or true negative nonhuman primate samples.

# 7   Appendices

## 7.1   Appendix I. Karnovsky's fixative protocol

Karnovsky's fixative 3% Glutaraldehyde, 2% Formalin in 0.1M $PO_4$ buffer, pH 7.2


Materials:

1. 3, 2 mL vials of 8% glutaraldehyde

2. Distilled water

3. 3.2 mL of 10% formalin solution

4. 0.1 M Phosphate buffer pH 7.4 (Prepare ahead):

    1. 20.209 g of Sodium Phosphate dibasic (mw: 268 g/mol)

    2. 3.3994 g of Sodium Phosphate monobasic (mw: 138 g/mol)

    3. Add distilled water to 1L

    4. Adjust pH with HCl or NaOH


Protocol:

1. Take the one vial of 8% glutaraldehyde (2 mL)

2. Add 0.67 mL of distilled water to make 2.67 mL of 6% glutaraldehyde.

3. Take 1.068 mL 10% formalin and dilute in 1.602 mL of $Na_2HPO_4$ Buffer to make 4% formalin solution.

4. Add 6% glutaraldehyde sol to 4% formalin solution 1:1 as needed.

5. Keep solutions separate until needed, any leftover mix can be saved at 4 ˚C for 1 month in a dark container.

## 7.2    Appendix II. Double centrifugation with Sheather's sugar flotation solution

Materials:

1.  Two small plastic containers
2.  Cheesecloth square (4 layers)
3.  Tongue depressor
4.  20 mL test tube; ensure that the top of the test tube is not cracked.
5.  Bamboo sticks
6.  Cover slip
7.  Slide
8.  Swing bucket centrifuge

Protocol:

1.  Label the test tube and slide with your sample numbers.
2.  Weigh 1 gram of sample in the plastic container.
3.  Use up to 15 mL of tap water to mix your sample. Be sure to mix it well to create a homogenous sample.
4.  Set the cheesecloth over the second container and strain the sample. Use a small amount of water to rinse the first container.
5.  Squeeze the cheesecloth to remove as much sample as possible.
6.  Transfer the sample to the test tube. Use a small amount of water to rinse the container to recover all the sample. The total quantity of water should not exceed 20 mL (max capacity of the test tube).
7.  Centrifuge at 500 x *g* for 5 minutes.
8.  Decant the supernatant.
9.  Add approximately 5 mL Sheather's sugar solution (1/4 of the test tube) and stir until the pellet has dissolved using a bamboo stick.
10. Place the test tube in the centrifuge and fill with Sheather's sugar solution – avoid creating bubbles. Continue to fill until there is a reverse meniscus on the surface.
11. Place a coverslip on the test tube and press down gently to create a seal.

12.     Centrifuge again 500 x *g* for 5 minutes.

13.     Allow the sample to sit 10 minutes for the eggs to rise to the cover slip. There is no maximum time period set. However, the cover slip must be removed and read prior to crystallization of the sugar (3-6 hrs).

14.     Gently remove the coverslip from the test tube and place it on a microscope slide, at an angle initially to avoid trapping bubbles of air.

15.     Complete the analysis of the slide using a light microscope 10x magnification.

## 7.3 Appendix III. Standard Operating Procedure for saliva collection

Purpose:

This SOP describes the procedure for collecting saliva from *C. sabaeus*.

Materials:

1. Extra-small chemical free braided cotton rolls (2)
2. 3 mL syringe (1)
3. Individually labeled zip top bags (1 per study subject)
4. Ice packs
5. Cooler
6. 15 mL Falcon tubes
7. Refrigerated Centrifuge
8. Micropipette
9. Micropipette tips
10. Eppendorf 2mL tubes
11. Eppendorf storage box
12. Eppendorf labels

Protocol:

1. Collection must be during the morning hours when circadian cycle changes in the salivary glands ensure optimal salivary flow and composition.
2. Record study subject number, time, and date of sample collection.
3. First, place 1 cotton roll under the upper lip on either side of the labial frenum.
4. Second, place 1 cotton roll under the lower lip on either side of the labial frenum.
5. Leave the cotton rolls in for a maximum of 5 min.
6. Assess the salivary flow by checking how full the cotton roll is after 2 to 3 min.
7. If cotton rolls are not filling up, meaning that salivary flow is low, gently massage them against the attached gingiva, aiming for the coronal-cervical junction and the gumline and

the rest of the mucobuccal folds avoiding the area of the 3<sup>rd</sup> molar where Stensen's duct from the parotid gland opens.

8. For the last minute, gently rub the cotton rolls against the hard and soft palates as well as the top of the tongue.

9.  Gently remove the cotton rolls to avoid sloughing the mucosal lining and place them in the 3 mL syringes. Reconnect the plunger.

10. Place the labeled syringe in the prelabeled zip top bag and place it on ice or at 4 ˚C immediately.

11. Process the saliva within 2-3 hrs of collection.

12. Take the plunger off the syringe and place it, plunger end upwards, into a 15 mL falcon tube.

13. Centrifuge at 1500 x $g$ at 4 °C for 20 min.

14. Remove the syringe containing the now dry cotton rolls and discard in biohazard bin.

15. Using a micropipette estimate the total amount of saliva collected.

16. Transfer the saliva into a pre labeled 2 mL Eppendorf tube and record the quantity as well as any other observations on consistency or color.

17. Add equal amounts of saliva conservation mix (1:1) (**Appendix IV**).

18. Freeze at -20 ˚C (for short term use up to 2 weeks) or -80 ˚C.

19. Avoid multiple freeze-thaw cycles.

## 7.4 Appendix IV. Saliva conservation mix

Saliva conservation mix (2x)

Materials

1. cOmplete™ Protease Inhibitor Cocktail Tablet, EDTA -free- (Roche ®) (2 tablets)

2. ProClin® (Sigma – Aldrich©) (100 µL)

3. PBS sterile (20 mL)

4. One 50 mL Falcon tube

5. Vortex

6. 100 µL Pipette and tips

7. 20 mL Pipette aid


Protocol:

1. Mix 2 tablets of protease inhibitor (cOmplete ™ Protease Inhibitor Cocktail Tablet, EDTA - free - Roche ®) in 20 mL of PBS.

2. Add 100 µL of ProClin® (Sigma - Aldrich©).

3. Mix by vortexing until tablets have dissolved completely.

4. Store frozen -20˚ C until ready to use.

5. Defrost at 4 C˚

6. Add to saliva samples 1:1

## 7.5 Appendix V. List of brand names and commercial addresses used

1. BMJ-III, Jiangsu, China
2. Olympus DP73 microscope, Tokyo, Japan
3. Roche ®, Mannheim, Germany
4. Sigma-Aldrich®, MO, USA
5. Dremel®, Mount Prospect, IL, USA
6. Misonix, NY, USA
7. Bio-Rad® CA, USA
8. Balzer Union, Balzers, Liechtenstein
9. Zeiss Auriga, Oberkochen, Germany
10. BD Microlance™, Fraga, Huesca, Spain
11. Spartan Chemical Company Inc, Ohio, USA
12. Virox® Technologies Inc. Ontario, Canada
13. Envigo® Teklad 8773 primate biscuits, Indiana, USA
14. Lixit® water valves, California, USA
15. Neologic solutions©, South Carolina, USA
16. Sanitron© ultraviolet water purifiers, Altlantic Ultraviolet Corporation®, New York, USA
17. Avidity Science©, Wisconsin, USA
18. Droncit®, Kansas, USA
19. Valbazen®, Michigan, USA
20. Bioiniche Pharma USA LLC, IL, USA
21. AnaSed® Akorn, Inc., IL, USA.
22. Richmond™, NC, USA
23. BD Luer-Lok tip, NJ, USA
24. Falcon™, NY, USA
25. Noromectin®, Newry, Co. Down, Northern Ireland
26. Thermo Fisher Scientific, MA, USA
27. Novusbio™ Novus Biologicals LLC, CO, USA
28. Abcam®, Cambridge, UK

29. Amersham™ Imager 600 (GE Healthcare®, IL, USA

30. Pharmacia Biotech AB®, Uppsala, Sweden

31. GE Healthcare®, New Jersey, USA

32. Promega, Madison, WI, USA

33. AB SCIEX©, CA, USA

34. Nikkyo Technos Co., Ltd, Tokyo, Japan

35. LC Packings, A Dionex Company, Amsterdam, Netherlands

36. Applied Biosystems®/MDS Sciex, MA USA

7.6    Appendix VII. IACUC approvals for African green monkey samples

**IACUC Tissue/Sample Use Approval Letter**

**Approved:** IACUC Application for Collection of Samples and Tissues
**DATE:** 1/30/2018
**TO:** Dr. Patrick Kelly
**FROM:** Ross University Institutional Animal Care and Use Committee
**Project Title:** Immunodiagnostics for *Trichuris Trichiura*

The purpose of this memo is to verify that the Ross University Institutional Animal Care and Use Committee (IACUC) reviewed and granted approval of the above described form submission.

Sincerely,

**Dr. SREEKUMARI RAJEEV**
**Chair, Institutional Animal Care and Use Committee**

# AXION RESEARCH FOUNDATION
## Biomedical Research on the Central Nervous System

February 26, 2018

**IACUC #: AC18175**
(Please refer this number when
inquiring about this project)

PRINCIPAL INVESTIGATOR: Shervin Liddie, Ph.D.

RxGen Inc.
3 Science Park, 4ᵗʰ Flr
New Haven, CT  065111

TITLE OF PROJECT: *Evaluation of the safety profile of Adeno-Associated Virus (AAV) vectors
following single intravenous infusion dosing in African green monkeys*

CURRENT APPROVAL PERIOD:  02/23/18  TO  02/22/19
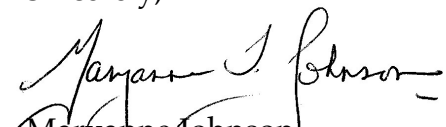
The IACUC Office granted approval for the use of animals in the project referenced
above on 02/23/18.  Please attach a copy of this approval to the approved protocol for
your files.

According to Federal Regulations, approval of this protocol is effective for one year and
approval may be renewed twice.  If the project will extend past three years another new
protocol application will be required for IACUC review.  Prior to the expiration of
initial approval and each of the two renewal periods, you will be asked to submit a
short progress report if you wish approval extended for an additional year.  If you do
not renew approval, you will be unable to use or order any animals under this project.

The Axion Research Foundation / St. Kitts Biomedical Research Foundation, has an
approved Animal Welfare Assurance number, A4384-01, from the Office of Laboratory
Animal Welfare, National Institutes of Health, effective May 20, 2020.

If you have any questions, or require any additional assistance, please do not hesitate to
call me.

Sincerely,

Maryanne Johnson
Committee Secretary

## 7.7    Appendix VIII. IRB approvals for human samples

# EXEMPTION CERTIFICATION

MEMO:     Katalina Cruz, DDS, MSc
                PhD Candidate

FROM:      Darryn Knobel, BVSc, MSc, MRCVS, PhD
                Institutional Review Board Chair

SUBJECT:   Exemption Certification for Protocol No. 18-10-EX

DATE:       June 14th, 2018

The Chair of the IRB determined that your project entitled: *Immunodiagnostics for Trichuris Trichiur,* meets federal criteria to qualify as an exempt study under 45 CFR 46.101(b)(4).

Because the study has been certified as exempt, you will not be required to complete continuation or final review reports. However**, it is your responsibility to notify the IRB prior to making any changes to the study**. Please note that **changes made to an exempt protocol may disqualify it from exempt status and require an expedited or full review.**

The IRB Office will hold your exemption application for six years. Before the end of the sixth year, you will be notified that your file will be closed and the application destroyed. If your project is still ongoing, you will need to contact the IRB Office upon receipt of that letter and follow the instructions for completing a new exemption application.

If you have questions or need additional information, please contact the Doris M. Castellanos at 401-1330 or dcastellanos@rossvet.edu.kn.

**Darryn Knobel**, BVSc MSc PhD MRCVS Dipl. ACVPM
**Chair, RUSVM Institutional Review Board**
**Professor of Epidemiology and Population Health**
**Director, Center for Conservation Medicine and Ecosystem Health**

# EXEMPTION CERTIFICATION

MEMO:      Patrick Kelly, PhD
           Professor Small Animal Medicine

FROM:      Darryn Knobel, BVSc, MSc, MRCVS, PhD
           Institutional Review Board Chair

SUBJECT:   Exemption Certification for Protocol No. 18-11-EX

DATE:      August 1st, 2018

The Chair of the IRB determined that your project entitled: *Immunodiagnostics for Trichuris Trichiur,* meets federal criteria to qualify as an exempt study under 45 CFR 46.101(b)(4).

Because the study has been certified as exempt, you will not be required to complete continuation or final review reports. However**, it is your responsibility to notify the IRB prior to making any changes to the study**. Please note that **changes made to an exempt protocol may disqualify it from exempt status and require an expedited or full review.**

The IRB Office will hold your exemption application for six years. Before the end of the sixth year, you will be notified that your file will be closed and the application destroyed. If your project is still ongoing, you will need to contact the IRB Office upon receipt of that letter and follow the instructions for completing a new exemption application.

If you have questions or need additional information, please contact the Doris M. Castellanos at 401-1330 or dcastellanos@rossvet.edu.kn.

**Darryn Knobel**, BVSc MSc PhD MRCVS Dipl. ACVPM
**Chair, RUSVM Institutional Review Board**
**Professor of Epidemiology and Population Health**
**Director, Center for Conservation Medicine and Ecosystem Health**

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# 9 Annexes

## 9.1 Supplementary table 1

Supplementary table 1. Proteins identified in *Trichuris trichiura* egg extract (EE) proteome by LC-MS/MS using ProteinPilot v 4.5. and Gene Ontology database.

| Description (alphabetical order) | Acc. No. WormBase | % Cov. | Peptides (95%) | MW (KDa) | Signal peptide | Molecular function | Biological process | subcellular location |
|---|---|---|---|---|---|---|---|---|
| 14-3-3 protein | A0A077YXJ9 | 17.9 | 7 | 33 | - | protein domain specific binding | unknown | unknown |
| 4 hydroxybutyrate coenzyme A transferase | A0A077ZC52 | 2.6 | 1 | 50 | - | transferase activity | acetyl-CoA metabolic process | unknown |
| 40S ribosomal protein S12 | A0A077ZFS5 | 51.9 | 6 | 14 | - | structural constituent of ribosome, ribonucleoprotein, ribosomal protein | translation | unknown |
| 40S ribosomal protein S14 | A0A077YZK6 | 16.0 | 2 | 15 | - | structural constituent of ribosome, ribonucleoprotein, ribosomal protein | translation | ribosome |
| 40S ribosomal protein S16 | A0A077ZGK9 | 10.3 | 2 | 17 | - | structural constituent of ribosome | translation | ribosome |
| 40S ribosomal protein S18 | A0A077ZDK7 | 17.1 | 2 | 17 | - | RNA binding, structural constituent of ribosome, ribonucleoprotein, ribosomal pro | translation | ribosome |
| 40S ribosomal protein S21 | A0A077ZE43 | 29.4 | 2 | 9 | - | structural constituent of ribosome | translation | ribosome |
| 40S ribosomal protein S24 | A0A077YXX2 | 9.1 | 1 | 14 | - | structural constituent of ribosome | unknown | translation |
| 40S ribosomal protein S28 | A0A077ZNE6 | 32.8 | 2 | 7 | - | structural constituent of ribosome | translation | ribosome |
| 40S ribosomal protein S3a | A0A077ZK25 | 6.9 | 1 | 29 | - | structural constituent of ribosome, ribonucleoprotein, ribosomal protein | unknown | cytoplasm |
| 40S ribosomal protein S7 | A0A077YW19 | 6.6 | 1 | 19 | - | structural constituent of ribosome | translation | ribosome |
| 40S ribosomal protein SA | A0A077YZD4 | 42.6 | 13 | 34 | - | structural constituent of ribosome, ribonucleoprotein, ribosomal protein | ribosomal small subunit assembly, translation | cytoplasm |
| 60 kDa SS A:Ro ribonucleoprotein | A0A077Z1S1 | 1.9 | 1 | 72 | - | RNA binding, Ribonucleoprotein | unknown | unknown |
| 60S ribosomal protein L11 | A0A077YX23 | 13.7 | 2 | 21 | - | structural constituent of ribosome | translation | ribosome |
| 78 kDa glucose regulated protein | A0A077Z8G8 | 22.6 | 12 | 72 | 1 to 18 | ATP binding | unknown | unknown |
| Acetyltransferase component of pyruvate dehydrogenase c | A0A077Z0S4 | 1.9 | 1 | 44 | - | dihydrolipoyllysine-residue acetyltransferase activity | pyruvate metabolic process | mitochondrial matrix |
| Actin | A0A077ZE37 | 55.6 | 35 | 41 | - | ATP binding | unknown | unknown |
| Actin 5C | A0A077WW9 | 53.7 | 29 | 41 | - | ATP binding | unknown | unknown |
| Actin-depolymerizing factor 2, isoform c | A0A077Z0N1 | 24.4 | 7 | 35 | - | actin binding | actin filament depolymerization | actin cytoskeleton |
| Acyl carrier protein | A0A077ZC49 | 16.2 | 2 | 16 | - | carrier of the growing fatty acid chain in fatty acid biosysthesis. | fatty acid biosysthesis and methabolism, lipid biosysth | unknown |
| Adenosylhomocysteinase | A0A077ZF21 | 8.3 | 4 | 47 | - | adenosylhomocysteinase activity, NAD binding, hydrolase | one-carbon metabolic process, S-adenosylhomocystein | unknown |
| Adh short and Glycos transf 1 and Glyco trans 4 4 domain c | A0A077Z6T2 | 4.6 | 3 | 81 | - | GDP-Man:Man1GlcNAc2-PP-Dol alpha-1,3-mannosyltransferase activity | protein glycosylation | unknown |
| ADH zinc N and ADH N domain containing protein | A0A077Z4S4 | 11.5 | 3 | 39 | - | oxidoreductase activity | unknown | unknown |
| ADP ribosylation factor 1 | A0A077ZIF5 | 9.3 | 6 | 87 | - | GTP binding | cell redox homeostasis | unknown |
| ADP ribosylation factor protein | A0A077Z8H5 | 17.7 | 2 | 14 | - | structural constituent of ribosome, ribonucleoprotein, ribosomal protein | translation | ribosome |
| ADP, ATP carrier protein, heart:skeletal muscle | A0A077ZJS2 | 3.7 | 1 | 35 | - | transmembrane transporter activity | unknown | mitocondrion inner membrane, integral component of the membrane |
| Aldo ket red domain containing protein | A0A077ZDK2 | 1.7 | 3 | 269 | - | oxidoreductase activity | unknown | unknown |
| Alpha-1,4 glucan phosphorylase | A0A077YWK8 | 20.3 | 14 | 101 | - | glycogen phosphorylase activity, linear malto-oligosaccharide phosphorylase activ | carbohydrate metabolic process | unknown |
| Aminopeptidase | A0A077Z146 | 3.7 | 3 | 110 | - | aminopeptidase activity, metallopeptidase activity, zinc ion binding | unknown | unknown |
| ATP dependent (S) NAD(P)H hydrate dehydratase | A0A077Z337 | 28.0 | 9 | 36 | - | ADP-dependent NAD(P)H-hydrate dehydratase activity, ATP binding, ATP-depende | Lyase | unknown |
| ATP dependent RNA helicase cgh 1 | A0A077ZMY4 | 7.4 | 3 | 50 | - | ATP binding, helicase activity, nucleic acid binding | unknown | unknown |
| ATPase inhibitor protein | A0A077Z2I0 | 8.7 | 1 | 14 | - | ATPase inhibitor activity | negative regulation of ATPase activity | mitocondrion |
| Bravo FIGEY and I-set and fn3 and Ig 3 domain con taining | A0A077Z439 | 4.4 | 3 | 142 | - | unknown function | unknown | integral component of membrane |
| BTB | A0A077YV88 | 3.3 | 1 | 58 | - | unknown function | unknown | unknown |
| Calmodulin | A0A077Z9J0 | 9.6 | 1 | 18 | - | calcium ion binding | unknown | unknown |
| Calponin like protein | A0A077YZI3 | 31.3 | 3 | 16 | - | unknown function | unknown | unknown |
| Calreticulin | A0A077ZC09 | 20.5 | 8 | 47 | 1 to 17 | chaperone, calcium ion binding, unfolded protein binding | protein folding | endoplasmic reticulum |
| Calsequestrin | A0A077ZEY0 | 16.4 | 5 | 49 | 1 to 21 | calcium ion binding | unknown | unknown |
| Carbonyl reductase (NADPH) 1 | A0A077ZE80 | 23.5 | 5 | 31 | - | unknown function | unknown | unknown |
| CBM 14 and TSP 1 domain containing protein | A0A077ZDE6 | 4.7 | 2 | 41 | - | chitin binding | chitin metabolic process | extracellular region |
| CBM 14 domain containing protein | A0A077Z111 | 46.7 | 38 | 96 | - | chitin binding | chitin metabolic function | unknown |
| CBM 14 domain containing protein | A0A077Z8B3 | 28.4 | 18 | 79 | - | chitin binding | chitin metabolic process | unknown |
| CBM 14 domain containing protein | A0A077ZFK2 | 1.2 | 2 | 192 | - | chitin binding | chitin metabolic process | extracellualr region |
| Chaperonin protein heat shock protein60 | A0A077ZIE8 | 28.0 | 11 | 63 | - | ATP binding | protein folding | cytoplasm |
| Chaperonin protein heat shock protein60 | A0A077ZIE8 | 9.8 | 4 | 63 | - | ATP binding | protein refolding, stress response | cytoplasm |
| Chloride intracellular channel exc 4 | A0A077Z6X1 | 3.5 | 1 | 34 | - | unknown function | unknown | unknown |
| Collagen alpha (Iv) chain | A0A077Z512 | 1.2 | 2 | 172 | - | extracellular matrix structural constituent | unknown | collagen trimer |
| Complement component 1 Q subcomponent-binding pro te | A0A077ZC92 | 28.1 | 3 | 22 | - | unknown function | unknown | mitochondrial matrix |
| Copper:zinc superoxide dismutase | A0A077Z0Y6 | 11.4 | 2 | 17 | - | metal ion binding, superoxide dismutase activity | unknown | unknown |
| Cuticle collagen 14 | A0A077Z745 | 4.6 | 1 | 32 | - | structural constituent of cuticle | unknown | collagen trimer, integral component of membrane |
| Cystatin | A0A077Z143 | 30.7 | 3 | 13 | - | cysteine-type endopeptidase inhibitor activity | unknown | unknown |
| Cystatin | A0A077Z255 | 18.5 | 5 | 27 | 1 to 17 | cysteine-type endopeptidase inhibitor activity, peptidase activity, hydrolase, prote | unknown | unknown |
| Cystatin | A0A077ZLF4 | 32.0 | 4 | 16 | 1 to 17 | cysteine-type endopeptidase inhibitor activity, protease inhibitor, Thiol proteae in | unknown | unknown |
| Cystatin | A0A077ZLD5 | 22.4 | 2 | 13 | 1 to 18 | protease inhibitor, Thiol protease inhibitor, cysteine-type endopeptidase inhibitor | unknown | unknown |
| Cystatin domain containing protein | A0A077Z0W3 | 35.7 | 3 | 11 | - | cysteine-type peptidase inhibitor activity | unknown | intracellular |
| Cytosol aminopeptidase | A0A077Z3I7 | 23.8 | 10 | 54 | - | aminopeptidase activity, manganese ion binding, metalloexopeptidase activity | unknown | cytoplasm |
| Dihydrolipoyl dehydrogenase | A0A077Z970 | 9.6 | 3 | 50 | - | oxidoreductase, dihydrolipoyl dehydrogenase activity, electron transfer activity, fl | cell redox homeostasis (3D model) | cell |
| Dipeptidyl peptidase family | A0A077ZBQ2 | 5.4 | 3 | 91 | - | serine-type peptidase activity | unknown | integral component of membrane |
| Disorganized muscle protein 1 | A0A077ZFM9 | 26.0 | 7 | 38 | - | unknown function | unknown | unknown |
| DUF290 domain containing protein | A0A077Z5X5 | 23.9 | 4 | 15 | 1 to 19 | unknown function | unknown | extracellular region or secreted |
| DUF290 domain containing protein | A0A077Z6V7 | 61.5 | 8 | 13 | - | unknown function | unknown | extracellular region or secreted |
| DUF290 domain containing protein | A0A077Z8H2 | 43.5 | 20 | 18 | 1 to 19 | unknown function | unknown | extracellular region or secreted |
| DUF290 domain containing protein | A0A077ZDA2 | 7.0 | 1 | 14 | 1 to 22 | unknown function | unknown | extracellular space |
| DUF290 domain containing protein | A0A077ZLE4 | 52.1 | 9 | 11 | - | unknown function | unknown | extracellular region or secreted |
| DUF3588 domain containing protein | A0A077Z2E1 | 1.7 | 3 | 161 | - | unknown function | regulation of transcription, DNA-templated | nucleus |
| ECH domain containing protein | A0A077Z1N9 | 44.8 | 17 | 31 | - | catalytic activity | metabolic process | unknown |
| Ectonucleotide pyrophosphatase:phosphodiesterase | A0A077ZCH4 | 2.2 | 1 | 55 | - | catalytic activity | unknown | integral component of membrane |
| Ectonucleotide pyrophosphatase:phosphodiesterase | A0A077Z1S7 | 1.6 | 1 | 79 | - | hydrolase activity, metal ion binding, nucleic acid binding | unknown | unknown |
| EF hand family protein | A0A077Z8X9 | 4.4 | 1 | 35 | 1 to 19 | calcium ion binding | unknown | unknown |
| Elongation factor 1-alpha | A0A077YYL7 | 33.5 | 12 | 51 | - | GTPase activity, GTP binding, translation elongation factor activity | Protein biosynthesis | cytoplasm |
| Elongation factor 2 | A0A077Z0M0 | 6.5 | 5 | 95 | - | GTPase activity, GTP binding, translation elongation factor activity | Protein biosynthesis | unknown |
| Endothelin converting enzyme 1 | A0A077ZLF3 | 21.3 | 6 | 41 | - | metalloendopeptidase activity | unknown | unknown |
| Enolase | A0A077YX57 | 44.5 | 27 | 50 | - | Magnesium ion binding, phosphopyruvate hydratase activity | glycolitic process | unknown |
| Epididymal secretory protein E1 | A0A077Z0I4 | 43.4 | 28 | 46 | 1 to 23 | intracelular cholesterol transport | unknown | unknown |
| Eukaryotic aspartyl protease superfamily | A0A077Z4E5 | 12.7 | 3 | 34 | 1 to 23 | hydrolase, protease, aspartic-type endopeptidase activity | unknown | unknown |
| Fatty acid binding protein | A0A077ZB79 | 15.7 | 2 | 14 | - | lipid binding | transport | unknown |
| Fructose-bisphosphate aldolase | A0A077Z6Y9 | 21.1 | 9 | 41 | - | Lyase, fructose-bisphosphate aldolase activity | glycolysis | unknown |
| Fumarate hydratase, mitochondrial | A0A077YZK2 | 14.2 | 6 | 50 | - | fumarate hydratase activity | fumarate metabolic process, tricarboxylic acid cycle | unknown |
| Galectin | A0A077ZG03 | 50.7 | 27 | 32 | - | carbohydrate binding | unknown | unknown |
| Galectin | A0A077ZIX8 | 39.6 | 25 | 32 | - | carbohydrate binding | unknown | unknown |
| Glucose-6-phosphate isomerase | A0A077ZIX8 | 18.1 | 7 | 63 | - | isomerasa, glucose-6-phosphate isomerase activity | gluconeogenesis, glycolytic process | unknown |
| Glucosylceramidase | A0A077Z0B4 | 5.4 | 2 | 61 | 1 to 18 | Glycosidase, Hydrolase, glucosylceramidase activity | sphingolipid metabolic process | unknown |
| Glyceraldehyde-3-phosphate dehydrogenase | A0A077ZHV3 | 56.1 | 66 | 38 | - | Oxidoreductase, glyceraldehyde-3-phosphate dehydrogenase (NAD+) (phosphoryla | glycolytic process, glucose metabolic process | unknown |
| GSHPx domain containing protein | A0A077ZJY1 | 8.2 | 2 | 42 | - | glutathione peroxidase activity | response to oxidative stress | unknown |
| GST C and GST N domain containing protein | A0A077ZC56 | 10.7 | 5 | 43 | - | glutathione transferase activity | unknown | unknown |
| GTP binding protein SAR1b | A0A077Z704 | 11.9 | 2 | 21 | - | GTP binding | intracelullar protein transport and vesicle mediated tra | endoplasmic reticulum and golgi aparatus. |
| Heat shock 70 kDa protein 9 (Mortalin) | A0A077ZM82 | 6.8 | 2 | 48 | - | ATP binding | Stress response | unknown |
| Heat shock protein 70 | A0A077Z8E4 | 20.1 | 21 | 130 | - | Structural constituent of the ribosome, L-malate dehydrogenase activity | Stress response, carbohydrate metabolic process,malat | unknown |
| Heat shock protein 90 | A0A077ZIF6 | 17.1 | 12 | 83 | - | ATP binding, unfolded protein binding | protein folding, response to stress | unknown |
| His Phos 1 domain containing protein | A0A077ZJA4 | 4.2 | 1 | 41 | - | unknown function | unknown | unknown |
| Histone domain containing protein | A0A077ZFV9 | 5.8 | 2 | 36 | - | DNA binding, protein heterodimerization activity. Core component of the nucleosor | nucleosome assembly | nucleus and chromosome |
| HSP20 domain containing protein | A0A077YWR4 | 9.5 | 2 | 22 | - | unknown function | unknown | unknown |
| HSP20 domain containing protein | A0A077Z9T3 | 17.7 | 4 | 44 | - | unknown function | unknown | unknown |
| HSP70 domain containing protein | A0A077ZM07 | 11.4 | 2 | 25 | - | ATP binding | unknown | unknown |
| Hypothetical FAD-dependent oxidoreductase YEL047C | A0A077YVJ4 | 3.8 | 2 | 57 | 1 to 24 | succinate dehydrogenase activity | unknown | unknown |
| Independent phosphoglycerate mutase | A0A077ZDB4 | 8.9 | 3 | 58 | - | manganese ion binding, phosphoglycerate mutase activity | glucose catabolic process | cytoplasm |
| Integrin beta | A0A077YZG6 | 2.8 | 1 | 49 | - | Integrin | unknown | integrin-mediated signaling pathway |
| Integrin beta | A0A077Z8U9 | 3.0 | 2 | 89 | - | Integrin | cell adhesion, integrin-mediated signaling pathway | memebrane, single-pass type 1, membrane protein |
| Intermediate filament protein ifa 1 | A0A077Z6U0 | 23.4 | 14 | 71 | - | unknown function | unknown | cytoskeleton, intermediate filament |
| KOW domain containing protein | A0A077YZ11 | 6.2 | 1 | 17 | - | structural constituent of ribosome | translation | large ribosomal subunit |
| Kunitz protease inhibitor | A0A077YUJ5 | 3.7 | 1 | 27 | 1 to 18 | hydrolase, protease, peptidase activity, serine type endopeptidase inhibitor activity | unknown | unknown |

| Protein name | Accession | A | B | C | Range | Molecular function | Biological process | Cellular component |
|---|---|---|---|---|---|---|---|---|
| L 3 hydroxyacyl coenzyme a dehydrogenase, short | A0A077Z595 | 33.8 | 8 | 35 | - | 3-hydroxyacyl-CoA dehydrogenase activity, NAD+ binding | fatty acid metabolic process | unknown |
| Laminin EGF and I-set and Laminin G 2 and Laminin B and i | A0A077Z9D4 | 4.3 | 9 | 305 | - | unknown function | *Lacks conserved residue(s) required for the propagati | unknown |
| Large subunit ribosomal protein 23 | A0A077Z4U6 | 14.3 | 1 | 15 | - | structural constituent of ribosome | translation | ribosome |
| Ldl recept a domain containing protein | A0A077Z2D1 | 6.4 | 5 | 86 | - | unknown function | unknown | integral component of membrane |
| LRR 5 domain containing protein | A0A077Z094 | 8.5 | 3 | 46 | 1 to 28 | unknown function | unknown | unknown |
| Macrophage migration inhibitory factor | A0A077Z686 | 41.8 | 9 | 20 | - | unknown function | unknown | unknown |
| Major sperm protein | A0A077Z265 | 21.5 | 3 | 22 | - | Central component in molecular interactions underlying sperm crawling. Forms an | unknown | cytoskeleton and endoplpasmic reticulum |
| Major sperm protein | A0A077Z897 | 4.8 | 1 | 25 | - | Central component in molecular interactions underlying sperm crawling. Forms an | unknown | cytoskeleton and endoplpasmic reticulum membrane |
| Major sperm protein | A0A077ZFS2 | 12.7 | 2 | 15 | - | Central component in molecular interactions underlying sperm crawling. Forms an | unknown | cytoskeleton |
| Major sperm protein | A0A077ZIQ5 | 14.0 | 3 | 19 | - | Central component in molecular interactions underlying sperm crawling. Forms an | unknown | cytoskeleton and endoplpasmic reticulum |
| Malate dehydrogenase | A0A077ZZW9 | 18.0 | 6 | 36 | - | Oxido reductase, L-malate dehydrogenase activity | carbohydrate metabolic process, malate metabolic process | unknown |
| Malic enzyme | A0A077Z5U2 | 28.0 | 13 | 63 | - | Oxidoreductase, malate dehydrogenase (decarboxylating) (NAD+) activity, metal ion b | unknown | unknown |
| Mediator of RNA polymerase II transcription subun it 22 | A0A077Z2H0 | 69.1 | 17 | 15 | 1 to 19 | unknown function | unknown | extracellular region or secreted |
| Mediator of RNA polymerase II transcription subun it 22 | A0A077Z5M8 | 24.8 | 2 | 12 | - | unknown function | unknown | extracellular space |
| Moesin:ezrin:radixin 1 | A0A077ZIT0 | 26.0 | 12 | 556 | - | actin binding | unknown | Cytoskeleton |
| Muscle cell intermediate filament protein OV71 | A0A077ZJF5 | 5.9 | 3 | 64 | - | structural molecule activity | unknown | cytoskeleton, intermediate filament |
| Myoglobin | A0A077ZJ83 | 17.0 | 3 | 16 | - | heme binding, oxygen binding | unknown | unknown |
| Myosin tail family protein | A0A077Z6U9 | 2.5 | 4 | 226 | - | actin-binding, actin filament binding, ATP binding, microtubule binding, microtubul | microtubule-based movement | cytoskeleton, miosin complex |
| NADH dependent fumarate reductase | A0A077ZIT3 | 8.8 | 4 | 54 | 1 to 27 | succinate dehydrogenase activity | unknown | unknown |
| Nascent polypeptide associated complex protein | A0A077ZH89 | 12.4 | 2 | 25 | - | unknown function | unknown | nascent polypeptide-associated complex |
| NEDD8 activating enzyme E1 regulatory subunit | A0A077ZIC3 | 1.9 | 1 | 77 | - | ubiquitin-like modifier activating enzyme activity | unknown | unknown |
| Nucleobindin 1 | A0A077ZAK1 | 5.2 | 2 | 62 | - | calcium ion binding | unknown | unknown |
| Nucleoside diphosphate kinase | A0A077YZ73 | 46.4 | 6 | 17 | - | ATP binding, nucleoside diphosphate kinase activity | kinase, ATP-binding | ribosome |
| OV 16 antigen | A0A077ZPX5 | 36.4 | 6 | 20 | - | unknown function | unknown | unknown |
| Palm thioest domain containing protein | A0A077Z837 | 10.3 | 2 | 35 | - | palmitoyl-(protein) hydrolase activity | protein depalmitoylation | integral component of membrane |
| Paramyosin | A0A077Z8E1 | 38.6 | 30 | 101 | - | motor activity | unknown | unknown |
| PDZ domain containing protein | A0A077YWS0 | 9.6 | 2 | 36 | - | unknown function | unknown | unknown |
| Peptidase C1 domain containing protein | A0A077ZGN1 | 19.4 | 5 | 35 | - | cysteine-type peptidase activity | unknown | unknown |
| Peptidase M13 and Peptidase M13 N domain containi ng pro | A0A077ZJE5 | 24.1 | 14 | 81 | - | metalloendopeptidase activity | unknown | unknown |
| Peptidase M13 N domain containing protein | A0A077ZAS2 | 3.6 | 1 | 32 | - | metalloendopeptidase activity | peptide hormone processing | unknown |
| Peptidyl-prolyl cis-trans isomerase | A0A077Z7S3 | 5.9 | 1 | 23 | 1 to 20 | isomerase, rotamase, peptidyl-prolyl cis-trans isomerase activity | protein folding | unknown |
| Peptidyl-prolyl cis-trans isomerase | A0A077YZV4 | 27.8 | 3 | 19 | - | peptidyl-prolyl cis-trans isomerase activity | protein folding | unknown |
| Peroxiredoxin-2 | A0A077Z7M0 | 29.0 | 20 | 71 | - | peroxiredoxin activity | cell redox homeostasis | cell |
| Phosphoenolpyruvate carboxykinase GTP | A0A077ZNK7 | 16.9 | 3 | 22 | - | Kinase, GTP binding, kinase activity, phosphoenolpyruvate carboxykinase (GTP) ac | gluconeogenesis | unknown |
| Phosphoglucomutase | A0A077YY22 | 4.7 | 3 | 62 | - | intramolecular transferase activity, phosphotransferases, magnesium ion binding | carbohydrate metabolic process, Ligand: MAGNESIUM, | unknown |
| Phosphoglycerate kinase | A0A077Z3K7 | 25.4 | 9 | 45 | - | ATP binding, phosphoglycerate kinase activity | Glycolytic process | unknown |
| Poly-cysteine and histidine tailed protein isofor m 2 | A0A077Z5Q5 | 50.9 | 109 | 51 | - | unknown function | unknown | unknown |
| Polyubiquitin | A0A077ZCM7 | 58.9 | 8 | 69 | - | unknown function | unknown | unknown |
| Probable nuclear transport factor nuclear transp ort factor | A0A077YWY7 | 5.2 | 1 | 15 | - | unknown function | unknown | unknown |
| Profilin | A0A077YXS2 | 22.2 | 2 | 15 | - | actin binding | unknown | unknown |
| Proliferation associated protein 2g4 | A0A077YZ84 | 13.0 | 4 | 44 | - | unknown function | unknown | unknown |
| Propionyl coenzyme A carboxylase beta chain | A0A077Z4W0 | 14.3 | 6 | 60 | - | ligase activity | unknown | unknown |
| Propionyl-CoA carboxylase alpha chain, mitochondr ial | A0A077ZBT2 | 5.8 | 5 | 132 | - | Ligase, ATP binding, ligase activity, metal ion binding, oxidoreductase activity. | unknown | unknown |
| Proteasome subunit alpha type | A0A077Z826 | 4.2 | 1 | 26 | - | Hydrolase, Protease, Threonine protease, threonine-type endopeptidase activity | proteasome-mediated ubiquitin-dependent protein cat | nucleus and cytoplasm |
| Proteasome subunit alpha type | A0A077ZDP8 | 7.3 | 2 | 28 | - | threonine-type endopeptidase activity | ubiquitin-dependent protein catabolic process | nucleus and cytoplasm |
| Proteasome subunit beta type 2 | A0A077YZS4 | 8.3 | 2 | 24 | - | threonine-type endopeptidase activity | proteolysis involved in cellular protein catabolic proce | proteasome core complex |
| Protein asteroid | A0A077Z2C7 | 63.6 | 34 | 31 | 1 to 23 | unknown function | unknown | unknown |
| Protein clc protein cbr clc-4 protein cbr clc-1 cre clc-4 pro | A0A077ZPB0 | 7.3 | 1 | 19 | - | unknown function | integral component of membrane | membrane |
| Protein disulfide isomerase A6 | A0A077YWN0 | 5.1 | 2 | 47 | - | isomerase activity | cell redox homeostasis | cell |
| Protein disulfide-isomerase | A0A077ZJZ3 | 35.0 | 14 | 55 | 1 to 18 | protein disulfide isomerase activity | cell redox homeostasis | Endoplasmic reticulum |
| Protein disulfide-isomerase | A0A077ZLF1 | 35.9 | 15 | 56 | 1 to 16 | protein disulfide isomerase activity | cell redox homeostasis | unknown |
| Protein LSM14 A | A0A077Z0S5 | 3.5 | 1 | 52 | - | unknown function | unknown | unknown |
| Protein ttr | A0A077ZAY5 | 18.2 | 2 | 17 | 1 to 19 | unknown function | unknown | extracellular region or secreted |
| Purine nucleoside phosphorylase | A0A077YZ86 | 18.4 | 3 | 29 | - | purine-nucleoside phosphorylase activity, glycosyltransferase, transferase | nucleoside metabolic process | unknown |
| Putative heat shock protein | A0A077ZCH5 | 11.5 | 2 | 22 | - | stress response | unknown | unknown |
| Putative transforming growth factor-beta-induced protein | A0A077ZAA9 | 12.9 | 7 | 77 | 1 to 22 | unknown function | unknown | unknown |
| Pyruvate dehydrogenase component subunit beta mit ocho | A0A077ZCG5 | 9.5 | 3 | 49 | - | catalytic activity, metal ion binding | unknown | unknown |
| Pyruvate dehydrogenase E1 component subunit alpha | A0A077YYR5 | 2.4 | 1 | 42 | - | pyruvate dehydrogenase (acetyl-transferring) activity | acetyl-CoA biosynthetic process from pyruvate | intracellular membrane-bounded organelle |
| Ras protein Rab 11B | A0A077ZJ45 | 13.2 | 2 | 21 | - | GTPase activity, GTP binding | unknown | unknown |
| Ras protein Rab 1A | A0A077YZ16 | 32.6 | 7 | 23 | - | GTPase activity, GTP binding | unknown | unknown |
| Ras protein Rab 2 | A0A077Z693 | 12.7 | 2 | 24 | - | GTPase activity, GTP binding | unknown | unknown |
| Ras protein Rab 5C | A0A077YUU2 | 11.5 | 2 | 24 | - | GTPase activity, GTP binding | unknown | unknown |
| Receptor expression-enhancing protein | A0A077YZ42 | 25.4 | 2 | 21 | - | Receptor | unknown | membrane, membrane protein |
| Receptor expression-enhancing protein | A0A077ZFJ9 | 19.5 | 4 | 19 | - | Receptor | unknown | membrane |
| Receptor expression-enhancing protein | A0A077Z4Z4 | 18.1 | 2 | 19 | - | unknown function | unknown | membrane, multipass membrane protein |
| Receptor expression-enhancing protein | A0A077Z831 | 10.0 | 1 | 19 | - | unknown function | unknown | membrane, multipass membrane protein, integral component of the membrane |
| Retinal dehydrogenase 2 | A0A077YZA6 | 2.1 | 1 | 58 | - | oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NAE | unknown | unknown |
| Ribos L4 asso C and Ribosomal L4 domain containi ng p | A0A077Z5S6 | 3.1 | 1 | 45 | - | structural constituent of ribosome | translation | ribosome |
| Ribosomal L18p and Ribosomal L18 c domain contain ing p | A0A077ZPB6 | 42.7 | 13 | 36 | - | 5S rRNA binding, structural constituent of ribosome | translation | ribosome |
| Ribosomal L22e domain containing protein | A0A077YWZ8 | 19.3 | 2 | 16 | - | structural constituent of ribosome | translation | ribosome |
| Ribosomal L30 and Ribosomal L30 N domain containi ng pr | A0A077ZLC7 | 6.5 | 2 | 29 | - | unknown function | translation | ribosome |
| Ribosomal protein L15 | A0A077YZY9 | 17.3 | 4 | 29 | 1 to 16 | Ribonucleoprotein, ribosomal protein | unknown | ribosome |
| Ribosomal protein S17 | A0A077Z2N6 | 16.7 | 3 | 19 | - | structural constituent of ribosome, ribonucleoprotein, ribosomal protein | translation | ribosome |
| Ribosomal protein S5 | A0A077ZA98 | 10.4 | 2 | 26 | - | RNA binding, structural constituent of ribosome, ribonucleoprotein, ribosomal pro | translation | small ribosomal subunit |
| Ribosomal S19e domain containing protein | A0A077Z148 | 5.8 | 1 | 17 | - | structural constituent of ribosome | translation | ribosome |
| S10 plectin domain containing protein | A0A077ZHZ4 | 28.5 | 3 | 15 | - | unknown function | unknown | unknown |
| Sb:cb283 protein | A0A077Z4B7 | 4.7 | 2 | 60 | - | aminopeptidase activity, manganese ion binding, metalloexopeptidase activity | unknown | cytoplasm |
| Serpin domain containing protein | A0A077Z1Z4 | 5.0 | 2 | 43 | - | unknown function | unknown | extracellular space, integral component of the membrane. |
| Small heat shock protein | A0A077ZA52 | 14.0 | 2 | 19 | - | metal ion binding | stress response | unknown |
| Small heat shock protein | A0A077ZD68 | 9.4 | 1 | 19 | - | metal ion binding | stress response | unknown |
| Small heat shock protein | A0A077Z7R3 | 34.8 | 5 | 18 | - | Ribonucleoprotein | Stress response | unknown |
| Small nuclear ribonucleoprotein associated | A0A077YZJ6 | 4.1 | 1 | 21 | - | Ribonucleoprotein | unknown | unknown |
| Small nuclear ribonucleoprotein Sm D1 | A0A077ZAE6 | 16.5 | 1 | 13 | - | Ribonucleoprotein | mRNA processing, mRNA splicing, spliceosomal snRNP | nucleus |
| StAR-related lipid transfer protein 5 | A0A077YVV8 | 4.0 | 1 | 28 | - | bile acid binding, lipid binding | unknown | unknown |
| Succinate dehydrogenase [ubiquinone] flavoprotein subun | A0A077YWE3 | 3.4 | 2 | 71 | - | oxidoreductase, flavin adenine dinucleotide binding, succinate dehydrogenase (ubiq | electron transport chain, tricarboxylic acid cycle | mitocondrion |
| Succinate dehydrogenase cytochrome b560 subunit | A0A077YWQ6 | 4.4 | 1 | 20 | - | electron transfer activity, metal ion binding, succinate dehydrogenase activity | tricarboxylic acid cycle | integral component of membrane, succinate dehydrogenase complex |
| Superoxide dismutase [Cu-Zn] | A0A077Z345 | 69.9 | 12 | 15 | - | oxidoreductase, metal ion binding, superoxide dismutase activity | unknown | unknown |
| Surface antigen repeat family protein | A0A077Z0L6 | 0.5 | 3 | 213 | - | regulation of autophagy | unknown | extracellular space, region or secreted |
| Synaptobrevin-1 | A0A077Z458 | 32.2 | 3 | 13 | - | unknown function | vesicle-mediated transport | integral component of membrane |
| TAFII28 and Ribosomal L11 N and Ribosomal L11 dom ain | A0A077ZKU5 | 2.6 | 1 | 28 | - | ribonucleoprotein, ribosomal protein, protein heterodimerization activity, structu | transcription initiation from RNA polymerase II promc | nucleus and ribosome |
| T1L and CBM 14 domain containing protein | A0A077ZFY4 | 1.4 | 2 | 196 | 1 to 31 | chitin binding | chitin metabolic process | extracellular region or secreted |
| Trans 2 enoyl coenzyme A reductase | A0A077Z149 | 13.0 | 4 | 41 | - | oxidoreductase activity | unknown | unknown |
| Translationally controlled tumor protein | A0A077Z8S9 | 21.5 | 2 | 20 | - | unknown function | Belongs to the TCTP family. | unknown |
| Transthyretin-like protein 46 | A0A077Z9N4 | 42.6 | 12 | 16 | 1 to 18 | unknown function | unknown | extracellular region or secreted (extracellular space) |
| Trefoil and Glyco hydro 31 domain containing prot ein | A0A077Z243 | 2.9 | 2 | 104 | 1 to 19 | carbohydrate binding, hydrolase activity, hydrolyzing O-glycosyl compounds | carbohydrate metabolic process | unknown |
| Triosephosphate isomerase | A0A077ZC84 | 57.3 | 10 | 27 | - | Isomerase, triose-phosphate isomerase activity | gluconeogenesis, glycolytic process, pentose-phosphat | unknown |
| Tropomyosin | A0A077ZIM1 | 41.2 | 38 | 87 | - | unknown function | unknown | unknown |
| Troponin C | A0A077Z6A8 | 18.5 | 2 | 18 | - | calcium ion binding | unknown | unknown |
| Troponin I 2 | A0A077ZDB6 | 6.9 | 1 | 31 | - | unknown function | troponin complex | cytoskeleton |
| TSP 1 and CBM 14 domain containing protein | A0A077Z3K5 | 5.7 | 4 | 93 | 1 to 27 | chitin binding | chitin metabolic process | extracellular region or secreted |
| Tubulin alpha chain | A0A077ZI14 | 9.1 | 3 | 50 | - | GTPase activity, GTP binding, structural constituent of the cytoskeleton | microtubule-based process-Tubulin is the major const | cytoskeleton |
| Tubulin beta chain | A0A077ZJ06 | 9.5 | 3 | 52 | - | GTPase activity, GTP binding, structural constituent of the cytoskeleton | microtubule-based process-Tubulin is the major const | cytoskeleton |
| Tyrosinase domain containing protein | A0A077ZE14 | 22.2 | 6 | 53 | 1 to 20 | metal ion binding, oxidoreductase activity | unknown | unknown |
| Ubiquitin associated and SH3 | A0A077ZFQ2 | 6.4 | 2 | 37 | - | unknown function | unknown | unknown |

| Protein | Accession | | | | | Molecular function | Biological process | Cellular component |
|---|---|---|---|---|---|---|---|---|
| UDP glucose pyrophosphorylase | A0A077YYW4 | 15.7 | 6 | 53 | - | Nucleotidyltransferase, UTP:glucose-1-phosphate uridylyltransferase activity | UDP-glucose metabolic process | unknown |
| UDP glucuronosyltransferase ugt 58 | A0A077ZDR2 | 9.3 | 3 | 46 | - | Transferase, transferase activity, transferring hexosyl groups | Transmembrane helix | integral component of membrane |
| Vitellogenin N and VWD and DUF1943 domain contain ing p | A0A077ZE83 | 56.3 | 205 | 199 | 1 to 19 | lipid transporter activity | unknown function | unknown |
| WAP domain containing protein, SLPI-like [Source:UniProt | A0A077YYJ8 | 6.3 | 1 | 29 | 1 to 25 | peptidase inhibitor activity | unknown function | extracellualr region or secreted |
| WAP domain containing protein, SLPI-like [Source:UniProt | A0A077ZDD4 | 6.8 | 1 | 14 | 1 to 18 | peptidase inhibitor activity | unknown function | extracellualr region or secreted |
| Zf-C3HC4 and Lactamase B and zf-CCCH domain conta ining | A0A077Z6W1 | 6.6 | 3 | 69 | - | hydroxyacylglutathione hydrolase activity, metal ion binding | methylglyoxal catabolic process to D-lactate via S-lacto | unknown |
| Zf-CDGSH domain containing protein [Source:UniProtKB/ | A0A077Z600 | 34.1 | 2 | 10 | - | 2 iron, 2 sulfur cluster binding | unknown function | intracellular membrane-bounded organelle |
| Uncharacterized protein | A0A077YZ22 | 25.1 | 3 | 16 | 1 to 17 | GPI anchor binding | positive regulation of voltage-gated channel activity, re | Cell membrane, GPI-anchor |
| Uncharacterized protein | A0A077ZMT5 | 14.1 | 20 | 84 | - | lipid binding | unknown function | unknown |
| Uncharacterized protein | A0A077Z2P1 | 13.4 | 2 | 19 | 1 to 16 | unknown function | unknown | unknown |
| Uncharacterized protein | A0A077Z906 | 13.0 | 4 | 39 | 1 to 17 | unknown function | unknown | unknown |
| Uncharacterized protein | A0A077ZG91 | 32.3 | 3 | 14 | - | unknown function | unknown | integral component of membrane |
| Uncharacterized protein | A0A077Z4J9 | 5.2 | 2 | 26 | - | unknown function | unknown | integral component of membrane |
| Uncharacterized protein | A0A077Z2Q3 | 52.2 | 7 | 51 | 1 to 15 | unknown function | unknown | unknown |
| Uncharacterized protein | A0A077YWH5 | 3.0 | 1 | 30 | - | unknown function | unknown | unknown |
| Uncharacterized protein | A0A077ZJW0 | 12.8 | 1 | 11 | 1 to 24 | unknown function | unknown | unknown |
| Uncharacterized protein | A0A077ZNL3 | 18.6 | 3 | 65 | - | unknown function | unknown | unknown |
| Uncharacterized protein | A0A077YWV5 | 3.5 | 1 | 37 | 1 to 27 | unknown function | unknown | integral component of membrane |
| Uncharacterized protein | A0A077ZDH0 | 37.0 | 13 | 66 | - | unknown function | unknown function | integral component of membrane |
| Uncharacterized protein | A0A077Z544 | 48.8 | 19 | 34 | 1 to 23 | unknown function | unknown function | unknown |
| Uncharacterized protein | A0A077YXT2 | 16.1 | 10 | 70 | 1 to 18 | unknown function | unknown function | unknown |
| Uncharacterized protein | A0A077YYL1 | 21.8 | 6 | 28 | - | unknown function | unknown function | unknown |
| Uncharacterized protein | A0A077Z0R1 | 42.7 | 6 | 18 | 1 to 19 | unknown function | unknown function | unknown |
| Uncharacterized protein | A0A077YX18 | 20.4 | 10 | 33 | 1 to 18 | unknown function | unknown function | unknown |
| Uncharacterized protein | A0A077YXJ5 | 25.7 | 5 | 174 | - | unknown function | unknown function | unknown |
| Uncharacterized protein | A0A077ZCJ0 | 7.6 | 1 | 12 | - | unknown function | unknown function | unknown |