

Article

Molecular Characterization of a Functional Membrane-Associated Progesterone Receptor Component 2 (PGRMC-2) in Maturing Oocytes of the Human Parasite Nematode *Trichinella spiralis*: Implications to the Host-Parasite Relationship

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Abstract: We explored the hypothesis that progesterone direct effect on *Trichinella spiralis* might be mediated indeed by a new steroid-binding parasite protein. Our first results showed that Progesterone decreases the parasite molting rate. We amplify, isolated, cloned and sequenced the PGRMC2 sequence using specific primers from known species. Furthermore, we expressed the protein and developed an antibody to performance confocal microscopy, where detected expression of a putative P4-binding protein exclusively located at the oocyte and the parasite's cuticle. Expression of the PGRMC2 protein in these cells was also confirmed by western blot and flow cytometry. Molecular modeling studies and computer docking for the PGRMC2 sequence protein showed that PGRMC2 is potentially able to bind to progesterone, estradiol, testosterone, and dihydrotestosterone with different affinities. Furthermore, phylogenetic analysis clearly demonstrated that *Trichinella spiralis* PGRMC2 is related to a steroid-binding protein of another platyhelminths. Progesterone may probably act upon *Trichinella spiralis* oocytes probably by binding to PGRMC2. This research has implications in the field of host-parasite co-evolution as well as the sex-associated susceptibility to this infection. In a more practical matter, these results may contribute to design new drugs with anti-parasite effects.

Keywords: *Trichinella spiralis*; muscle larvae; sex steroids; oocytes; helminth; PGRMC; hormone receptors, progesterone.

1. Introduction

Trichinellosis is a reemerging and globally spread zoonotic disease caused by different species of the *Trichinella* genus. Trichinellosis is considered as the most common helminthic foodborne disease. Seroprevalence studies proved that there is a constant

exposure to *Trichinella* in Mexico, showing that a 4% seroprevalence remained constant from 2002 to 2012 (unpublished data). Furthermore, studies show that in Eastern Europe, prevalence can go up to 50% in rural areas due to the consumption of raw or poorly cooked pork. Trichinellosis is a chronic disease with a difficult diagnosis, treatment is often aimed at symptoms rather than using an anthelmintic therapy. In addition to the great health risk it constitutes, there are several livestock losses.

Trichinella complete its life cycle in one host. Once the host ingests under-cooked meat that contains muscle larvae (ML), these start to develop until they reach their adult phase, adults will begin to mate and females will give birth to new born larvae (NBL). These NBL disseminate through circulatory system until they reach skeletal muscle tissue, in which they invade and develop to the stage of muscle larvae.

During this process the parasite is exposed to several component of the host like human hormone and exist evidence that these hormones can affect the parasite's development and in turn apparently these parasites need the hormones to fully develop. An important group of hormones are steroids, specifically, sex steroids, such as progesterone (P₄), estradiol (E₂) and testosterone (T₄), which have an effect on the development of helminth parasites like *Taenia*. In the *Taenia solium* cysticerci, it has been found *in vitro* that treatment with P₄ herein increases both evagination and growth, thus demonstrating a direct effect of P₄ on the parasite [1,2]. In *Taenia crassiceps*, another related cestode, P₄ treatment increases parasite loads by 2-fold in both male and female host as compared to uninfected animals [3]. Furthermore, Hernandez-Bello *et. al.*, (2011), found that the addition of different sex hormones to *Trichinella* cultures had a negative or positive outcome depending on the hormone used. In this case, P₄ had a negative effect on the development of LM *in vitro* whereas testosterone seemed to have a slight positive effect on the molting process of the ML. Flow cytometry assays showed a specific binding of anti-PR antibody suggesting the presence of a PR-like or a putative progesterone-binding receptor from parasitic origin [4]. Also, P₄ inhibited the expression of a membrane protein, caveolin-1, that is implicated in the oocyte development of the ML [4]. Interestingly, caveolins are associated to nuclear and membrane steroid hormone receptors [5], as the progesterone receptor membrane component 1 and 2 (PGRMC-1 and PGRMC-2) [6]. The latter has been found in tissues related to the reproductive system of mammals but also found in some invertebrates. For example, *Caenorhabditis elegans* express Vem-1, an analogous protein to PGRMC-2 in mammals [7]. For helminth parasites, several sex hormone-receptor-related proteins, including PGRMC, small androgen receptor interacting proteins, progesterone-receptor associated p48 protein and progestin-induced protein, have been reported in *Schistosoma japonicum* EST [8,9]. Likewise, several nuclear receptors as well as thyroid-hormone-associated proteins were identified in *Schistosoma mansoni* EST [10,11]. Recently, Aguilar *et. al.*, (2018) had report a protein resembling to PGRMC membrane receptor in *Taenia solium* cysticerci. Indeed, the effects on evagination and growth showed by the parasite in presence of progesterone could be mediated by this receptor [1].

This kind of study about steroid binding proteins could help to expand the knowledge about *Trichinella* spp biology in terms of reproduction, differentiation and development, as well as generating possible pharmacological targets that could be used in anti-helminth drug therapy.

The aim of the present study was the identification of a novel steroid-binding protein in *T. spiralis*, resembling to PGRMC2, through DNA sequencing, recombinant protein expression, immunofluorescence, molecular modeling, docking analysis, and phylogenetical computational analysis.

2. Materials and Methods

2.1 Ethic statement

Animal care, experiments and protocols were evaluated and approved by the Experimental Animal Care and Use Committee of the Institute (permission number 2016-00123)

at the Instituto de Investigaciones Biomédicas adhering to the official Mexican standards (NOM-062-ZOO-1999). Experiments were authorized by Ethics and Research Committee and the Institutional Committee for the Care and Use of Laboratory Animals, Medicine Faculty, Universidad Autónoma de Nuevo León (registration number: MB14-007). Mexican regulations are in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institute of Health (NIH) of the USA. Mice were euthanized using anesthesia overdose (Sevorane®).

2.2 Parasite isolation

Trichinella spiralis was maintained in BALB/c mice by serial passage infections. The infective-stage ML were recovered from experimentally infected mice at 30 days post infection (pi) by standard pepsin-hydrochloric acid digestion method. Larvae were washed several times with phosphate-buffered saline (PBS) and 300 LM were used to infect BALB/c mice. Adult parasites from one-to-five days pi were obtained from small intestine of infected mice.

2.3 Nucleic acid isolation

Total RNA from ML and Adult parasites was obtained by homogenization using Trizol Reagent (Invitrogen) according to manufacturer's instructions. The RNA was previously with RNase-free DNase I for avoid DNA contamination. Quality of RNA was monitored by non-denaturing agarose gel electrophoresis and quantified by the absorbance at OD260/OD280. Samples were stored at -70°C prior to RT-PCR. Poly A+ RNA isolation from total RNA was obtained using Micro-FastTrack 2.0 kit (Invitrogen) following the instructions manual.

2.4 Primers

Different sets of primers were designed using Oligo 7. The F and R designation indicates forward and reverse direction. The detection of the Membranal component of the *Trichinella* progesterone receptor (Ts-PGRMC2) was carried out using the following set of primers: PGRMC2-F (5'-TGAACAACCTTCGACAGTATGAC-3'; PGRMC2-R (5'-CTGCAAGATCATCGTATGTATC-3'). Amplification of the carboxyl end of the PRMC protein was carried out using the following set of primers: PGRMC2-CT-F (5'-GAACAACCTTCGACAGTA-3'); PGRMC2-CT-R (5'-ACTGATCATCAACATCACAATCAG-3'). The 419-bp fragment from RNA 18S (GenBank, Accession # U60231) was amplifying using the 18STs-F (5'-ACGAAAGTTAGAGGTTTCGAA-3') and 18STs-R (5'-CCCTCTAAGAAGTGATCAGC-3') set of primers. The RACE reaction was carried out using the manufacturers own set of primers plus the following set: PGRMC2-F (5'-CACCGCCCATGAAACGACGCTAT-3'); PGRMC2-R (5'-CCTTGAAGCGTCCCTCCCGGCAAA-3').

2.5 RT-PCR and PCR conditions

Five hundred nanograms of RNA were used for the synthesis of cDNA using the SuperScript II enzyme (Invitrogen) under the following conditions: 45° C for 1 h and 70° C for 15 min to inactivate the enzyme. This cDNA was either frozen (-70°C) or used for the amplification using the previously mentioned primers. All PCR reactions were performed as follow: denaturing at 94°C for 5 min, followed by 30 cycles at 94°C for 45s, 56°C for 30s and 72°C for 45s; and a final extension at 72°C for 7 min. The 392-bp carboxyl end fragment of the PGRMC2 was amplified using the PGRMC2-CT-F/R pair of primers and the Platinum *Pfx* DNA Polymerase (Invitrogen) following the manufacturer instructions. For the first-strand cDNA synthesis of the 419-bp fragment from RNA 18S, the 18STs-R primer and 0.2 µg of total RNA was used following the described instructions for RT assay. PCR amplification was carried out using the 18STs pair of primers in the same conditions as above. All these PCR reactions were performed in 30 µl final volume, containing

20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl₂, 2 μ M of each dNTPs, 0.5 μ M of each pair of specific primers selected and 1.5 units of recombinant Taq DNA polymerase (Invitrogen). In all cases 5 μ l of PCR products was resolved in a 1-1.5% agarose gel with ethidium bromide and photographed under UV light

2.6 5' and 3' rapid amplification of cDNA end (RACE)

Synthesis of the 5'- and 3'-end of the Ts-PGRMC2 fragment was performed using 250 ng of mRNA from ML parasites and two specific primers (PGRMC2-F and PGRMC2-R). The RACE amplification was done using the GENE Racer kit (Life technologies) following the manufacturer's instructions. Briefly, 250 ng of mRNA from LM were treated to ligate the *GeneRacer RNA Oligo* to the 5' end of the mRNA and then reverse-transcribed using the *SuperScript™ III RT* and the *GeneRacer Oligo dT Primer* following the manufacturer's instructions (Life Technologies). The 5'- and the 3'-end cDNA were amplified using the PGRMC2-R and the *GeneRacer™ 5'* pair of primers and the PGRMC2-F and the *GeneRacer™ 3'* Primer pair of primers respectively. The products amplified were purified using the Wizard SV Gel and PCR Clean-Up kit. Subsequently, the selected fragments were cloned into the pCR 4-TOPO® vector (Life Technologies) and then sequenced by LAMBAMA services.

2.7 Sequence analysis

The DIALING software was used for analysis of sequence alignments (<http://dialing.gobics.de/>). "Translate tool" software website (<https://web.expasy.org/translate/>) was used for the predicted amino acid sequence. DNA similarity and protein sequences were analyzed by FASTA3 (Pearson and Lipman, 1988) at the European Bioinformatics Institute website (EBI) (<http://www.ebi.ac.uk/fasta33/>) and BLAST programs (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>, Altschul et al., 1990). Transmembrane domains searching was performed on TMHMM software (<http://www.cbs.dtu.dk/services/TMHMM/>). Theoretical protein molecular weight was determined by https://web.expasy.org/compute_pi.

2.8 Expression and purification of CTNT recombinant protein

The 387-bp fragment encoding the C-terminal region of PGRMC2 of *T. spiralis* protein was cloned and expressed into pET-102 TOPO following the manufacturer instructions (Life Technologies). Briefly, One shot TOP 10 chemical competent *Escherichia coli* was transformed with the plasmid bearing the C-terminal region of PGRMC2 of *T. spiralis* (Ts-PGRMC2). Candidates were analyzed by enzymatic digestion assays using *Hind III* and *Xba I* restriction enzymes (BioLabs). The right clones were used for expression in *E. coli* BL21 Star (DE3) bacteria. Expression of the Ts-PGRMC2 was induced using 0.5 mM IPTG (Sigma) in 10 ml of bacteria culture at 0.5 optical density (O.D.₆₀₀) during 6 h. The soluble and insoluble extract were obtained following the instructions of the manufacturer (Invitrogen) and the proteins were electrophoresed in 15% SDS-polyacrylamide gels. The purification of the Ts-PGRMC2 recombinant protein was performed under denaturing conditions using Ni-NTA agarose (Qiagen) and then dialyzed against decreasing urea solutions. The integrity of the purified protein was verified by 8% SDS-PAGE acrylamide gel and its concentration was determined by Quick standard Bradford method (BioRad).

2.9 Polyclonal Anti-PGRMC2 Serum Production

Five BALB/c mice of 8-week-old were immunized i.p. with 4 μ g of the Ts-PGRMC2 purified protein plus Aluminium Hydroxide Gel (Alhydrogel) (Invivogen) in 50 μ l final solution. Immunization was performed every 2 weeks for 1 month. One week after the last immunization, mice were bled and the serum containing the anti-Ts-PGRMC2 antibodies was collected and stored at -20°C. Before using, the serum was diluted and

adsorbed for 1 h with bacterial extracts expressing non related Ts-PGRMC2 protein cloned in the same pET-102 vector.

2.10 Western blotting

Soluble and insoluble proteins from ML, Adult parasites and total extracts from heart of BALB/c mice were obtained according to the method described by Philipp *et. al.*, (1980) [12]. Briefly, the parasites were resuspended in Tris-P buffer with cComplete™ Protease Inhibitor Cocktail (Sigma) and disrupted by potter-homogenization. The lysates were centrifuged for 30 min at 11,000 × g, transferred to a new tube and resuspended in Tris-P buffer. Twenty-five µg of protein sample was boiled in Laemmli buffer with β-mercaptoethanol (β-ME) and separated by SDS-PAGE (15% polyacrylamide) (MiniPROTEAN II, Bio-Rad). Proteins were transferred into PVDF membrane (ThermoFisher Scientific) and blocked with TBS-T plus 5% nonfat dry milk (w/v) for 1 h at room temperature. The membrane was then incubated with anti-Ts-PGRMC2 mouse polyclonal antibody at 1:1,000 dilution in TBS-T for 1 h, followed by goat anti-mouse IgG secondary antibody conjugated to Horseradish Peroxidase (Pierce) at 1:10,000 dilution for 30 min. Chemiluminescent reagent (Pierce) was used to develop the reaction signal. Protein molecular weights were estimated by comparison with standard protein molecular weight markers (BioRad).

2.11 *Trichinella spiralis* cell isolation and flow cytometry

Trichinella spiralis disaggregated cells were obtained from ML by tissue disruption using a micropestle (Eppendorf, USA) until no more clumps were visible. Then, cells were centrifuged at 300 × g for 5 min and recovered in 100 µl of FACS buffer (phosphate-buffered saline (PBS) pH7.4, 2% SFB, 0.02% NaN₂) and 100 µl of PFA (Paraformaldehyde 4% in PBS 1x) was added. The cells were incubated at 4°C for 10 min with the following anti-mouse antibodies: Mac-1 and Mac-3, CD8-PE-Cy5, CD19-PE, CD3-FITC, CD4-FITC (BD Biosciences), and 20 mins for the mouse anti-Ts-PGRMC2 (1:100) and the anti-mPR (1:100. Santa Cruz Biotechnologies) antibodies [13]. Afterward, cells were incubated with the secondary antibody Alexa488 or FITC-conjugated goat anti-mouse (Zymed) and incubated at 4°C for 30 min in absence of light. Then, cells were washed twice and resuspended in 500 µL of staining buffer. All the samples were analyzed by flow cytometry using a FACS Calibur (BD, Biosciences) and data analyzed using the FlowJo© software.

2.12 Nuclei staining with diamidino-phenylindole (DAPI) of isolated cells.

Trichinella spiralis ML cells were collected, fixed in paraformaldehyde (Sigma) for 10 min, and permeabilized with 0.2% Triton X-100 (Merck) for 5 min. Then DAPI [0.5 µg/ml] was added to the fixed cells on the slide for 20 min in the dark. The slides were rinsed in PBS, then Fluoprep (bioMerieux) was added, a cover slip applied. For light microscopy applications, we used a Nikon Eclipse E600 microscope equipped with a Nikon DXM1200F CCD (Nikon Corp.; Tokyo, Japan). For immunofluorescence staining experiments were carried out using a Nikon Eclipse 80i microscope and a Nikon DXM1200C CCD (Nikon Corp.). For observation of DAPI filter was utilized for staining technique (Nikon Corp.). Image processing and analysis was carried out using Adobe Photoshop CS3 (Adobe Systems Inc., San Jose, CA, U.S.A) and Image Pro Plus 6.2 (Media Cybernetics, Bethesda, MD, U.S.A)

2.13 Confocal microscopy

Four micrometers cryocut sections of *T spiralis* ML and adults were fixed with 4% PFA for 30 min, washed, permeabilized and blocked for 30 min with RPMI medium containing 0.5% BSA and 5% FBS. After, the slides were incubated with mouse anti-Ts-PGRMC2 polyclonal antibody (1:500) and then incubated with (FITC)-conjugated goat anti-mouse antibody (Sigma) at 1:200 dilution. Control experiments were performed in absence of

mouse anti-Ts-PGRMC2 antibody. All samples were contrasted with 0.025% Evans Blue for 10 min to eliminate background fluorescence and examined in a Leica TCS SP5 confocal laser-scanning microscope (Leica Microsystems, Germany). The images were constructed using Leica Confocal Software.

2.14 Docking of testosterone and dihydrotestosterone to *Trichinella spiralis* membrane-associated progesterone receptor component 2 (Ts-PGRMC2)

Initial model generation was carried out with the cloned sequence for *Trichinella spiralis* PGRMC2 and submitting it to Rosetta Homology modelling [1]. Resulting models clustered close together for the selection of the best model. However, since the template is a PGRMC1 protein in complex with a heme group [14], these models were refined using UCSF Chimera-Modeller plugin in order to include the heme group [15,16]. Then they were submitted to energy minimization using Amber 2018 [17] and their quality was evaluated using Molprobit [18]. The highest quality model was selected to perform ligand docking. Blind docking was performed using Vina 1.1.2 [19] on a 8-core computer running Windows Server 2012. All ligands were obtained from the ZINC database and converted to PDBQT format using the GUI provided by Autodock Tools. The receptor was kept rigid during docking. Docking employed a grid of dimensions 40 × 40 × 40 with a 1 Å grid size. Exhaustiveness was always set to 1000. Analysis of the docking results was performed in UCSF Chimera. The results presented in Table are the best candidates selected from the consensus score the three best results.

2.15 Alignment and phylogenetic analysis

The *T. spiralis* PGRMC2 sequence was aligned with other 21 sequences from the GenBank database, including 12 sequences for vertebrates (mammals, birds, reptiles and fish) 3 for arthropods, 2 for nematodes and 4 for platyhelminthes. The alignment included 133 characters (amino acids) and was constructed using Clustal W [20] with default parameters and adjusted manually within Mesquite [21]. The genetic divergence among taxa was estimated using uncorrected “p-distance” with MEGA version 6 [22]. The tree was constructed with neighbor-joining method and the nodes of the tree were supported with 10,000 bootstrap replicates.

3. Results

3.1. PGRMC2 gene expression on *T. spiralis* muscle larvae

We found a predicted sequence in the genome of *Trichinella spiralis* that encodes for a protein that has similarity with PGRMC2 of other organisms (Gene Bank: KRY42019.1). This sequence has 705 pairs of bases, but it lacks the 5'-untranslated region (UTR) and it doesn't present a polyadenylation site characteristic of the RNA messenger. We performed a *in silico* transcription of this sequence which showed a hypothetical protein of 234 amino acids with an estimated molecular weight of 26.71 kDa. Also, we used the TMHMM tool to determine transmembrane sites in the aminoacidic sequence. This analysis showed a transmembrane site, as well as a cytoplasmic site and an intracellular site. Subsequently, an analysis of this sequence in the BLAST Conserved Domains platform showed a cytochrome b5-like Heme/Steroid binding domain between the 104-173 amino acids region (data not showed). In order to investigate the expression of the putative PGRMC2 of *Trichinella spiralis* a RT-PCR was performed using total RNA of ML and a pair of primers (PGRMC2-F and -R) which flanking the steroid binding domain. The electrophoretic analysis showed a 209 bp band that corresponds to the expected amplified band, however, another 290 bp band was observed. To discard a possible genomic DNA (gDNA)

contamination, the total RNA from LM was treated with DNase. Despite the enzyme treatment the faint 290 bp band still appeared. (Figure 1A).

3.2. 5'- and 3'- RACE for PGRMC2 of *T. spiralis*

To obtain the complete sequence of the transcribed PGRMC2 of *Trichinella spiralis* a RACE 5' and 3' assays were performed. This technique allows the amplification of the missing ends of the sequence. For the 5'- end sequence two fragments of 485 bp and 580 bp were found (Figure 1B); while four fragments of 321, 388, 434 and 858 bp for the 3'- end sequence were found (Figure 1C). After different alignments analysis of the sequences obtained, two different variants of the transcribed named 460 and 580 bp were constructed respectively (Figure 2). Both sequences allowed to obtain a new 694 nucleotide sequence. We performed a translation of this sequence and found that encoded for a 133 residues protein which contained the topological characteristics of PGRMC2 proteins including a transmembrane region, and a heme-binding domain (cytochrome b5-like Heme/Steroid binding domain) (Figure 2B and 2C) [23].

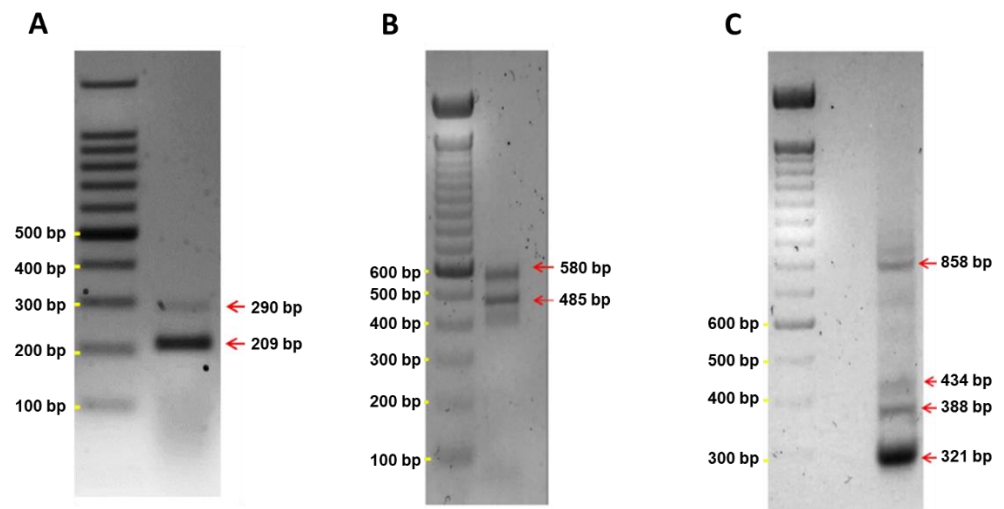


Figure 1. *Trichinella spiralis* PGRMC2 expression and sequencing. A) RT-PCR using total RNA of ML treated with Dnase. B) and C) Fragments amplified by the 5'- and 3'-RACE method, respectively. In all agarose gel, the numbers at the left indicate MW.

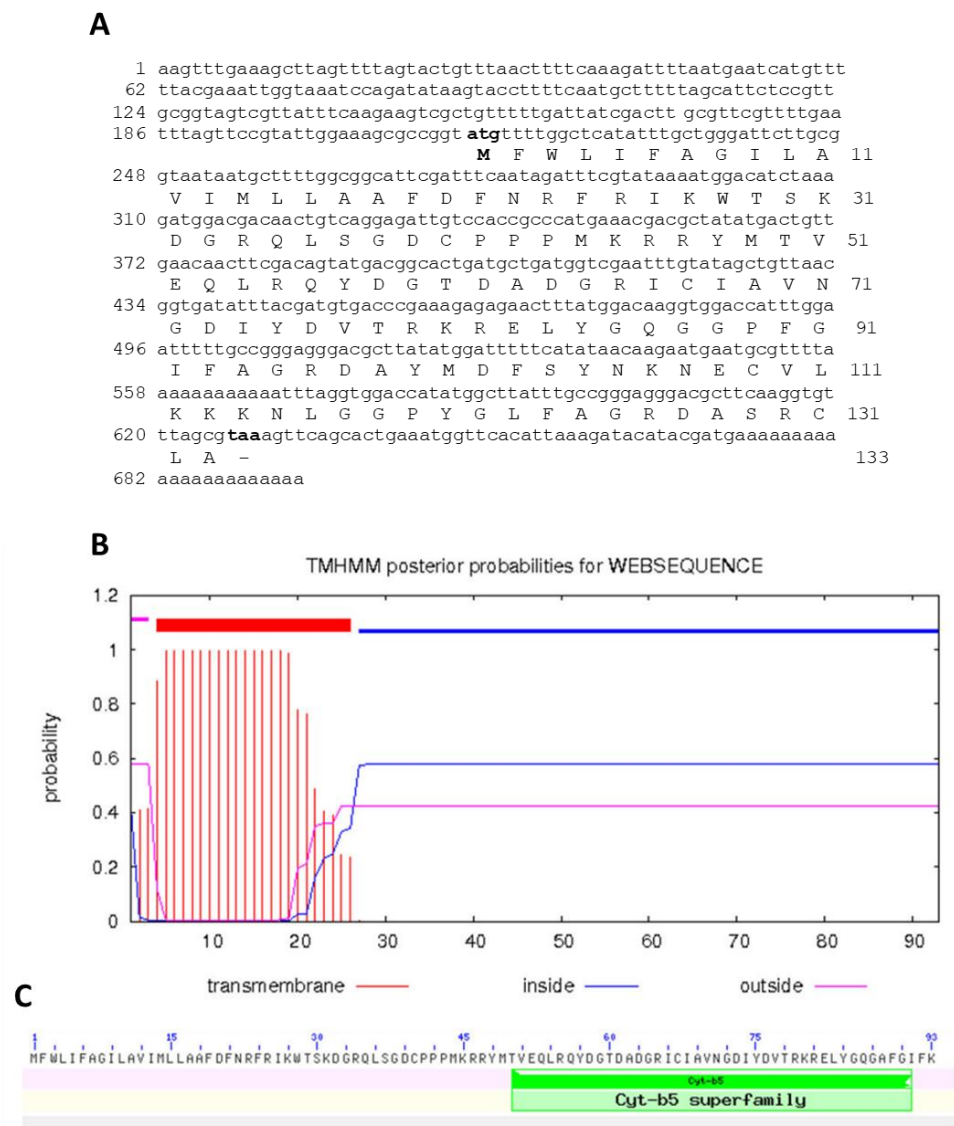


Figure 2. *Trichinella spiralis* PGRMC2 (Ts-PGRMC2) sequence. A) Nucleotide and amino acid complete Ts-PGRMC2 sequence. Numbers at the left and the right indicate nucleotide and amino acid positions, respectively. The length of the cDNA is 674-bp and the protein sequence is 133 amino acids. B) Transmembrane region analysis of the Ts-PGRMC2. C) Motif searching of acid complete Ts-PGRMC2 sequence.

3.3. Anti-PGRMC2-CT serum recognizes the recombinant and native protein

Later, in order to generate antibodies against the Ts-PGRMC2 protein for further immunolocalization analysis, the C-terminal end (133 residues) was expressed in the pET-102 vector. The highly expressed Ts-PGRMC2 recombinant protein was found in the soluble fraction (Figure 3A), then was purified and mixed with Alhydrogel (Invivogen) and then administrated to 8-week-old BALB/c mice. Before using the anti-Ts-PGRMC2 polyclonal serum, adsorption was carried out using bacterial lysates expressing non-related-Ts-PGRMC2 protein in the pET-102 vector, as a necessary step to eliminate possible antibodies recognizing Thioredoxin protein, the V5 epitope and 6X-His tail belonging to the vector (Figure 3B). In blots using extracts of BL21 Star DE3 bacteria expressing Ts-PGRMC2 protein, the polyclonal serum was able to recognize the 33 kDa Ts-PGRMC2 recombinant protein with high specificity, and unspecific binding was not detected. Subsequently soluble and insoluble proteins from ML were electrophoresed and transferred to nitrocellulose membrane (Figure 3C). The adsorbed anti-Ts-PGRMC2 serum was then incubated with the membrane and one single band of approximately 11.7 kDa was

observed in the soluble and insoluble fraction of ML lysates. This result was consistent with the theoretical molecular weight predicted for a 133-amino acid protein from the Ts-PGRMC2 coding sequence (Figure 3D).

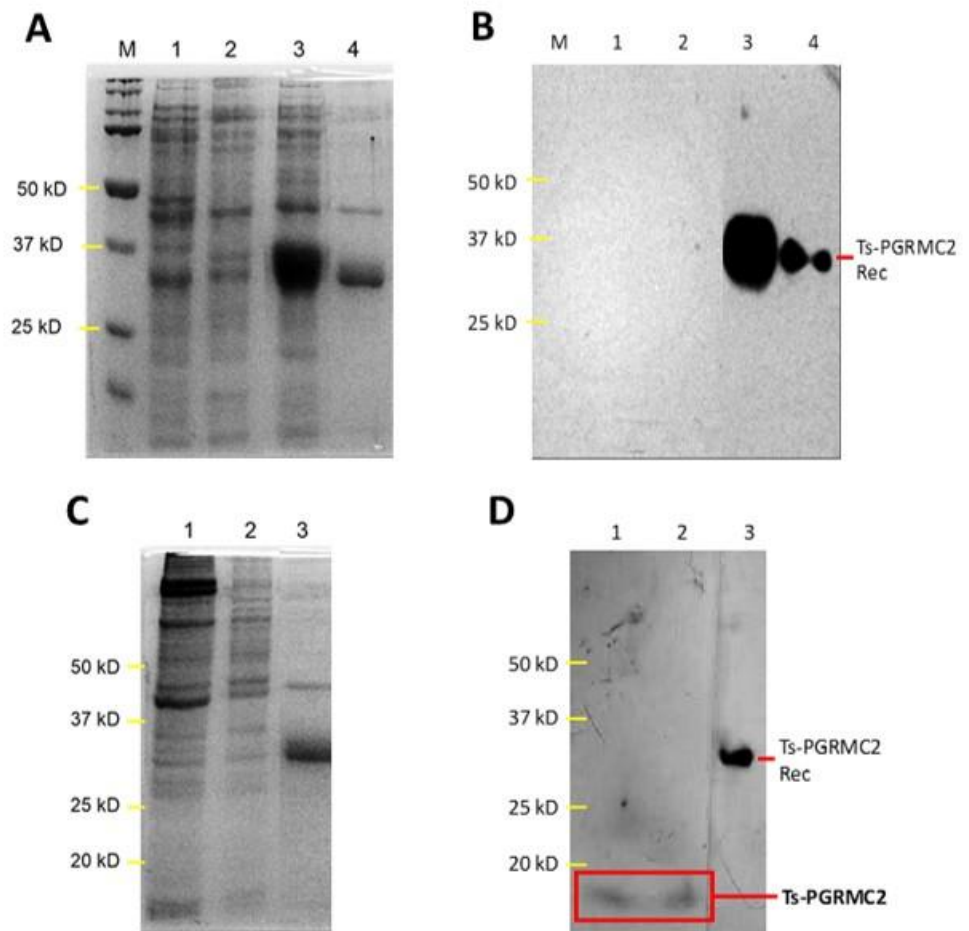


Figure 3. Western Blot of the recombinant (Ts-PGRMC2 Rec) and native (Ts-PGRMC2) *Trichinella spiralis* PGRMC 2 protein. A) Coomassie of lysate BL21 bacteria without (1) and with pET102 plasmid (2); induced IPTG bacteria (3) and purified recombinant protein (4) are showed. B) Western blot from transferred proteins using the anti-Ts-PGRMC2 antibody. C) *Trichinella spiralis* ML insoluble (1) and soluble (2) proteins; line 3 correspond to the Ts-PGRMC2 Rec protein. D) Western blot from transferred proteins using the anti-Ts-PGRMC2 antibody where the recombinant and native proteins are detected.

3.4. *Trichinella spiralis* single cells isolation

Once well characterized and identified the Ts-PGRMC2 protein, we designed the experiment for its localization in the parasite's cells. The first step was the isolation of single cells of *T. spiralis* as showed in figure 4. The photograph shows the complete parasite, before of the isolation process (Figure 4A). In the second picture (Figure 4B) the parasite was disrupting and show clumps and debris of pieces of parasite. Finally, in the third picture the isolated single cells of the parasite are depicted. It is interesting to note that, *Trichinella spiralis* cells are multinucleated and due to the size (10 μ m) it cannot be a complete parasite. In order to display and quantify the cells for further experiments, they were visualized stained with DAPI which allowed us to visualize the cell nuclei, but also an easy cell quantification. A representative example of these techniques (phase contrast, cell nuclei DAPI-staining) is shown in Figure 4.

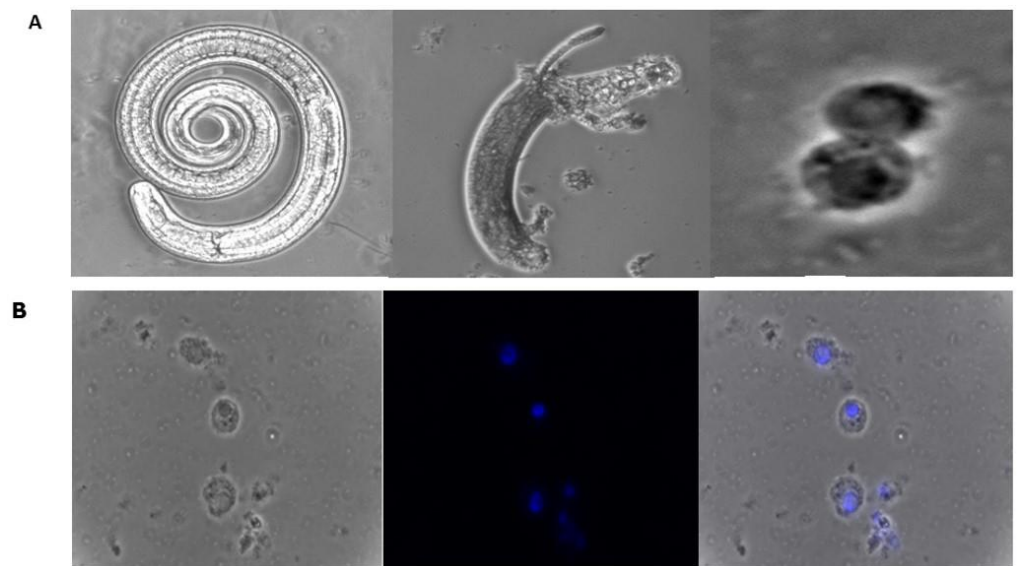


Figure 4. Imaging of the process of getting single cells from *Trichinella spiralis* after mechanical disaggregation. (A) *Trichinella spiralis* (first picture), pieces after disaggregation and (B) *Trichinella spiralis* isolated cells. Pictures were taken using an inverted microscope (Olympus, MO21, Tokyo) at 10X and 100X magnification. Disruption of parasite tissue and staining of primary cells. After isolation, primary cells were fixed and nuclei were stained with DAPI. Composed figure depicts stained cells with DAPI (produces blue color) of *Trichinella spiralis* cells.

3.5. Detection of the putative progesterone binding protein in *Trichinella spiralis* larvae by flow cytometry

To discard any cell contamination from the host (mice) in the *T. spiralis* single cell obtaining, the size and complexity of *T. spiralis* cells were settled. Flow cytometry analysis showed that *Trichinella spiralis* cells were different in size and complexity from mouse spleen cells (Figure 5A). In fact, parasite cells were approximately 3-fold smaller and exhibited less complexity than the mouse spleen cells (Figure 5B). In addition, parasite cells have no expression of the membrane markers CD3, CD19 and CD11b which are typically present in some types of mammalian leukocytes marker of all types of mammalian cells (Figure 5B).

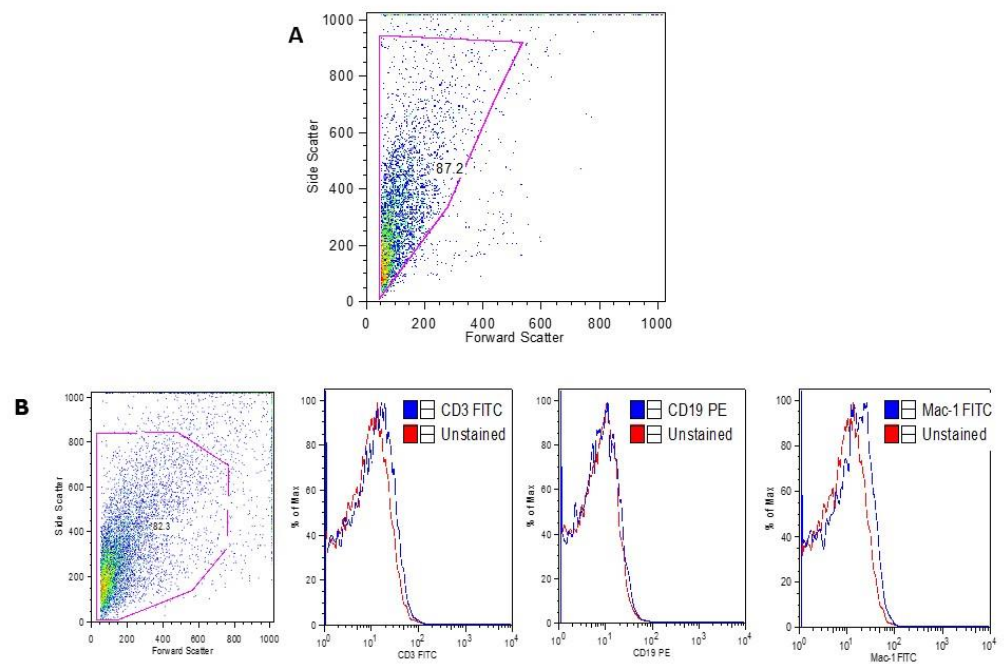


Figure 5. Forward/Side scatter of splenocytes and parasite isolated cells. (A) Spleen cells, (B) *Trichinella spiralis* cells were disaggregated by tissue disruption, washed twice with FC buffer and fixed using Lyse/Fix buffer (BD Biosciences). Non-host-contaminated parasite isolated cells. *Trichinella spiralis* cells were disaggregated by tissue disruption, and stained with anti-mCD3, anti-mCD19, anti-mCD11b and anti-mMac-3 antibodies.

Once we have confirmed no contamination by host cells in the single cell isolation of *T. spiralis* procedure, we carried on with the Ts-PGRMC2 native protein detection in cell's parasite. Before that, expression of mPR isoforms in isolated cells of *Trichinella spiralis* was analyzed by flow cytometry. We found that *Trichinella spiralis* do not express any mPR α isoform (mPR α and β) (data not shown). Interestingly, when we analyzed the expression of Ts-PGRMC2 after 24 hours stimulation with P4, the expression of this receptor was negatively regulated (Figure 6). Since the anti-Ts-PGRMC2 antibody was able to detect the native protein, we decide to investigate its localization in several stages of *T. spiralis* parasites.

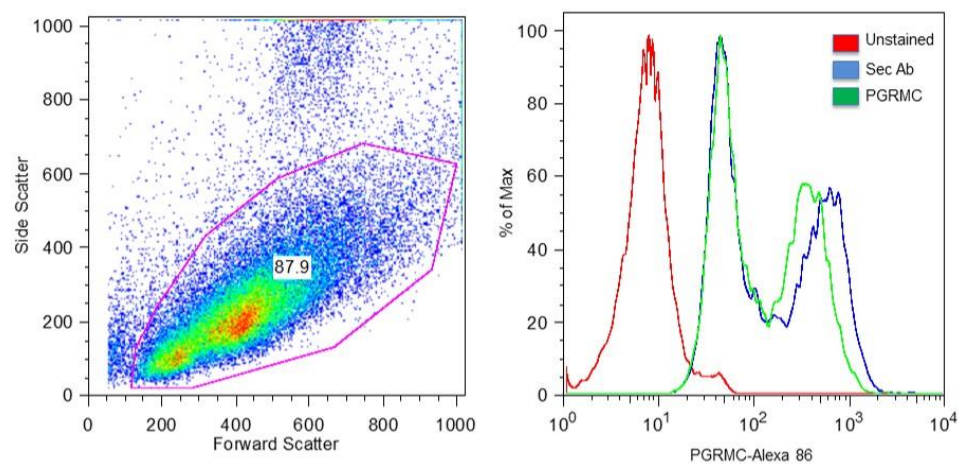


Figure 6. Specific detection of PGRMC in *Trichinella spiralis* larvae by flow cytometry. FACS analysis in larvae of *Trichinella spiralis* cells, showed the anti-Ts-PGRMC2 expression in both oocytes and cuticle. Left panel, size and complexity of

Trichinella spiralis cells. Right panel, Ts-PGRMC2 cell expression. Blue line corresponds to unspecific staining of the secondary antibody and green line corresponds to the specific PGRMC2 staining cells.

3.6. Expression and immunolocalization of PGRMC-Ts in *Trichinella spiralis* ML and Adults.

To determinate the Ts-PGRMC2 expression at different stages of the parasite, we obtained the total RNA from ML and adults from 1-, 3- and 5-days post-infection. The differences between the parasite's stages are mainly the developmental degree of oocytes and embryos in the parasite's uterus. Our results showed that Ts-PGRMC2 expression was constant without any change independently of the parasite stage, in contrast with caveolin, which expression increased in a dependent way of the oocyte and embryo development [24]. Afterwards, we investigate the Ts-PGRMC2 localization in these stages. Our result showed that fluorescence was present in the oocytes, embryos and the cuticle of all parasite stages, which reveals the Ts-PGRMC2 protein is equal expressed in the ML cells as well as cells from adult parasites from 1 to 5 days pi and did not alter its localization. (Figure 7). Likewise, *T. spiralis* parasites sections incubated only in the presence of the secondary antibody used as control, did not gave any positive signal, which confirmed that the experimental conditions were optimal for detecting exclusively parasite cells expressing Ts-PGRMC2 molecules and not false positive signals.

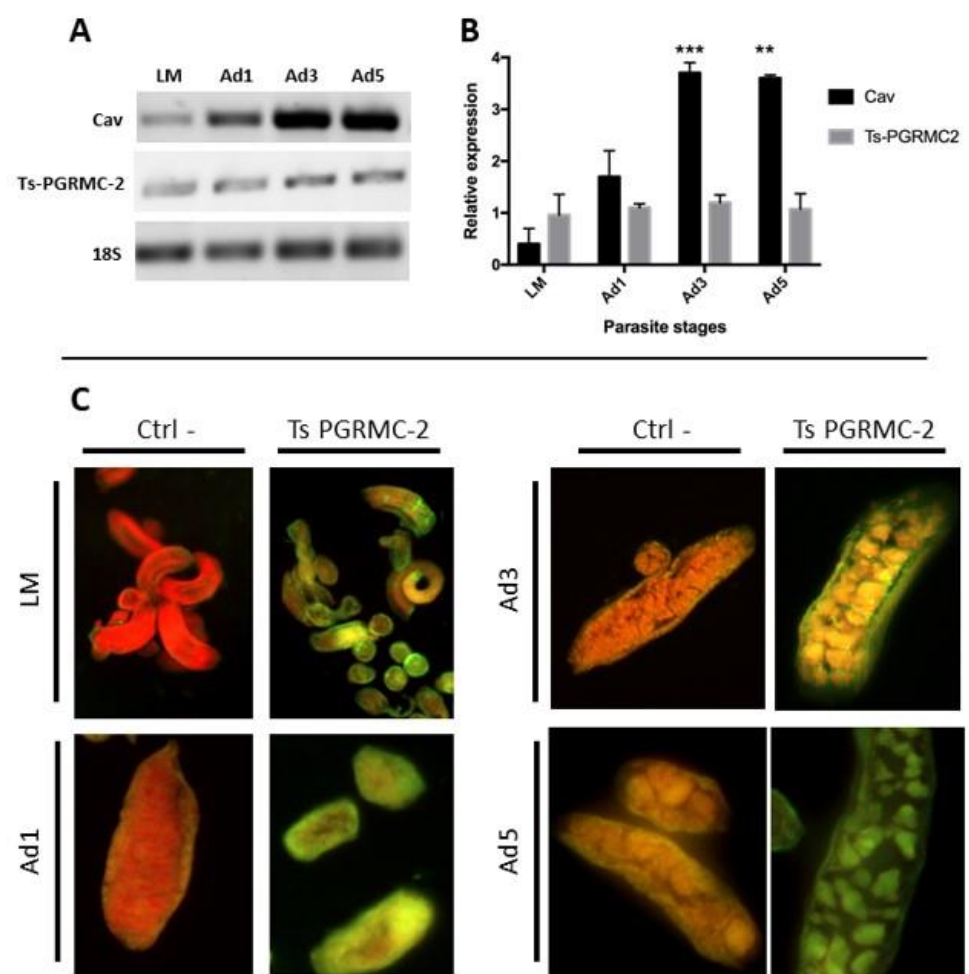


Figure 7. *Trichinella spiralis* PGRMC2 (Ts-PGRMC2) expression and immunolocalization. A and B, shows the RT-PCR of Ts-PGRMC2. The 18S RNA was used as reliable internal control and Cav-1 of *T. spiralis* (Cav) was used as a positive

control. C, Immunolocalization of Ts-PGRMC2; anti-Ts-PGRMC2 antibody and fluorescein isothiocyanate-conjugated goat anti-mouse antibody were used in a four micrometer sections from LM and adult parasites previously treated with Evans blue for contrast. Immunofluorescence in green and yellow, show a positive localization of Ts-PGRMC2 protein.

3.10. Docking of PGRMC to several steroids

In further Ts-PGRMC2 protein characterization, an *in silico* model was generated and is quite in line with the template despite having a 42% homology. Most of the residues known to interact with the heme group in the PGRMC1 structure are identical in *T. spiralis* PGRMC2 (Figure 8A). As mentioned above if we remove the heme from our model and dock it using the same protocol as for the ligands, the binding site has a high affinity (Figure 8A). The resulting model contains a heme group partially buried and contributing significantly to the binding of all of the ligands tested. Interestingly, for every case, the three best results for each ligand were in contact with the heme group on the surface of the protein (Figure 8B). Notably, P₄ is the most tightly bound ligand followed by dihydrotestosterone and testosterone. Given that residue TYR149 (numbering based on the whole sequence cloned) provides the fifth coordination to the heme-iron, interactions with the other side of the heme group are seemingly blocked. It is unknown if Ts-PGRMC2 is able to dimerize as its template (*Homo sapiens* PGRMC1), but the interactions with the ligands tested in the present work would block a similar interaction (Table 1).

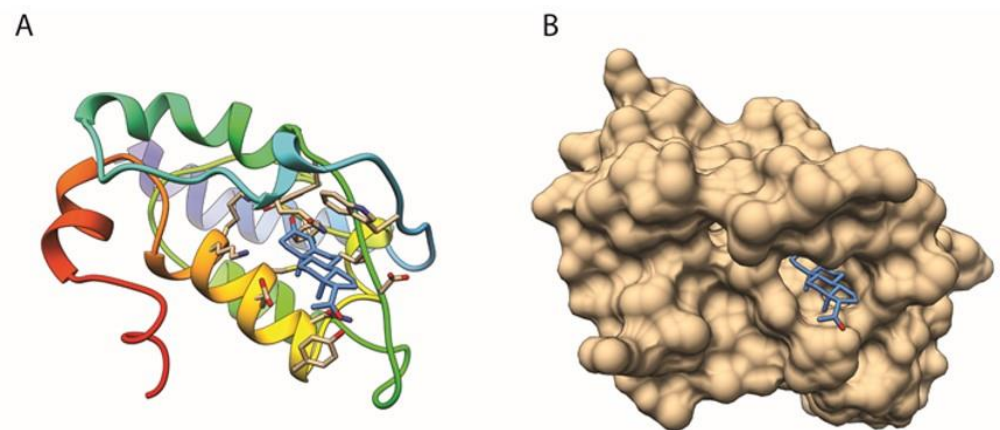


Figure 8. Model for *Trichinella spiralis* PGRMC2 and its docking to P₄. The model for Ts-PGRMC2 lacks a binding pocket for a heme group but binds testosterone tightly (A). A TRP residue forms part of the binding site. (B) A surface representation of our model shows that the binding site is partially buried inside the protein.

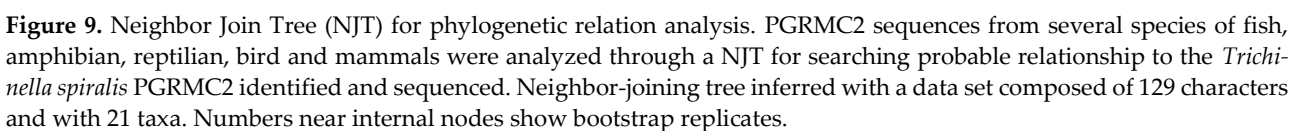
Table 1. Docking of several hydrophobic compounds to PGRC2. The data presented show the best result for each compound.

Ligand	Interaction energy (kcal/mol)	Score
Testosterone	-9.1	-78.8 ¹
Dehydroepiandrosterone	-7.9	-77.55 ¹
Beta-estradiol	-7.7	-76.87 ¹
Cortisol	-7.7	-78.16 ¹
Estriol	-7.7	-75.42
4,5 alpha-Dihydrotestosterone	-7.5	-73.41 ¹
Progesterone	-7.5	-71.47 ¹
Cholesterol	-7.2	-75.46 ¹
Corticosterone	-7	-75.46 ¹
Aldosterone	-6.8	-68.3 ¹
Albendazole	-6.3	-72.5 ¹
Linoleate	-6.1	-92.97 ²

Linoleic acid	-6	-95.95 ²
Dodecanoate	-5.7	-78.88 ²
Oleate	-5.7	-87.73 ²
Sterate	-5.7	-84.78 ²
Myristate	-5.6	-80.23 ²
Palmitate	-5.6	-80.23 ²
Decanoate	-5.4	-71.57 ²
Octanoate	-5	-64.14 ²

3.11. Phylogenetic analyses of Ts-PGRMC2

The neighbor-joining tree obtained in the current study, yielded that the progesterone receptor component of *T. spiralis* is nested within a cluster that includes other Platyhelminthes as *Schistosoma haematobium* and *Schistocephalus solidus* with a strong bootstrap support of 69%. The neighbor-joining tree, also placed together the three sequences of arthropods and the 12 sequences of vertebrates are spread out in different cluster (Figure 9). Finally, the progesterone receptor component sequences of the swine (*Sus scrofa*), and human conformed a cluster in the tree with 100% bootstrap support, and this cluster is very divergent to progesterone receptor component of *T. spiralis* (Figure 9).



The genome of *Trichinella spiralis* was sequenced in 2011 by Mitreva, *et. al.*, by "shotgun genome sequencing" method [25]. From these fragments a construction of the whole genome of the nematode was carried out. These genes opened the possibility of using analytical tools to detect the presence of different coding sequences that could contain the parasite genome. In these analyses, made with bioinformatics tools such as EAnnot, SNAP and FgenesH (which are based on algorithms that find preserved regions, such as the sites

of polyadenylation), we found a predicted sequence for PGRMC2 gene [25–27]. Using a set of primers, we were able to complete the non-coding regions (UTR's) at the 5' and 3' ends of this predicted sequence, including to the polyadenylation site. We able to detect 2 different variants of the PRMC2-Ts transcript (Figure 2). Despite our Ts-PGRMC2 sequence was shorter than the previously reported sequence, both variants encode for the same 93 amino acid sequence. One explanation is similar to reported by Leel *et al* in 2004, where a cDNA encoding a 75 amino acid truncated form were cloned. This clone contained the amino terminal coding sequence (transmembrane domain) and a cytoplasmic sequence does not present in PGRMC1, maybe as result of frame shift [28] or an alternative "splicing" phenomenon, known as "Alternative Polyadenylation" (APA). This latter process consists of the variation of the 3' UTR of some RNA transcripts and could be presented in 2 forms, 1) an alternative edition in the coding region (CR-APA), which affects the protein that will be expressed or 2) an edition in the UTR 3' region (UTR-APA). It has been described that the process of UTR-APA, can have effects on the amount of the expression of a protein, since in the UTR 3', are regions that can define the stability of the transcript, as well as the location of the final product [29]. Coupled with this, it has been described that this phenomenon is presented in a differential way depending on the cells of the organism, in addition to its stage of development [30]. Interestingly, this phenomenon is present in oocytes of *Xenopus* and *Caenorhabditis elegans* germline [30,31].

About the topology of the Ts-PGRMC2 amino acid sequence transmembrane and intracellular domains were found (Figure 1). This is consistent with the topology of the membrane-associated progesterone receptor (MARF) family, which Ts-PGRMC2 belongs [6,32,33]. On the other hand, BLAST analyses, to find preserved domains on this protein; showed that it possesses a site binding to steroids at the end carboxyl terminal (50 to 90 aa). The antibody generated against the terminal carboxyl of Ts-PGRMC2 was able to recognize the native protein in both the single cells and the cross sections in the parasites in a specific manner. The localization of this protein was found on the cuticle but mainly in oocytes and embryos. That suggest a possible implication in the oocyte differentiation and embryo development since its expression is constant. Unfortunately, PGMCR2 has been poorly studied in mammals and other organisms, compared with the closely-related PGRMC1 and the reports about its function are scarce [33]. However, in mammals, PGRMC1 is found in cells of reproductive organs like ovary and testicle. PGRMC1 mediates the antiapoptotic effects of progesterone in rat granulosa cells; on the other hand, experiment demonstrated that PGMRC1 protein are involved in the acrosome reaction in human sperm [34]. Also, the free-living nematode, *Caenorhabditis elegans*, possesses a VEM-1 gene that shared 37% amino acid identity with PGRMC1, which is implicated in neuron guidance and axon formation as well as neurons in the nematode ventral midline [7]. Terzaghi *et. al.* in 2016, reported that PGRMC1 participates in late events of both mammalian mitosis and oocyte meiosis [35]. All these data, support that idea of the role of Ts-PGRMC2 for oocyte maturing and parasite offspring.

We previously reported that cholesterol start to accumulate in the immature oocyte membrane of *T. spiralis* reaching the maximum accumulation in the late embryos at 3 days pi [24]. Cholesterol is an important molecule implicated in the nematode development, as demonstrated Shim *et al.*, 2002, where proliferation and differentiation of the *Caenorhabditis elegans* germ line is affected under cholesterol starvation [36]. PGRMC1, is implicated in the cholesterol regulation; because some proteins like Insig-1 and SCAP has been described to direct interact with PGRMC1 for cholesterol process in COS7 cells where transcription of sterol regulatory element (SRE)-regulated genes are involved [23,37]. The presence of Ts-PGRMC2 in the oocytes and early and late embryos, could suggest its participation on cholesterol regulation together with other proteins like caveolin-1 of *T. spiralis* which is up regulated during both oocyte and embryo development and cholesterol enrichment in the membrane [24]. Indeed, has been reported co-precipitation of

PGRMC1/VemaA using anti-caveolin antibody in bovine ovary [38] which suggest that PGRMC2/Caveolin-1/cholesterol ensemble could participate in several roles in the oocytes and embryos of *T. spiralis*.

Finally, the docking analysis, showed that PGMRC2 is able to bind progesterone but to a better extent testosterone. This may partly explain why male host presents and increased parasitic load compared with female host [39]. Phylogenetic analysis confirm that the sequence obtained belongs to PGRMC2 protein family.

5. Conclusions

It was shown that the parasite *Trichinella spiralis* has 2 variants of the transcribed Ts-PRMC2. These 2 variants of the transcribed have the same reading frame, so they encode for the same protein of 93 AA, however, there could be a phenomenon of alternative polyadenylation in the edition of these transcripts or a possible PGRMC2 isoform.

The recombinant protein produced proved to be efficient for the immunization of mice, since this allowed the obtaining of a polyclonal antibody that recognized the protein Ts-PGRMC2 in the soluble and insoluble fractions of *Trichinella spiralis* in conditions Denaturant (Western Blot) as well as in native conditions (immunofluorescence).

The native protein of *Trichinella spiralis*, presented a molecular weight of 11.7 kDa, both in the soluble protein fraction and in the insoluble.

The membrane receptor Ts-PGRMC2 is located in the membrane of the germ cells of the parasite, which probably confers a relevance on the development, as well as the differentiation and development of their offspring.

6. Patents

This section is not mandatory but may be added if there are patents resulting from the work reported in this manuscript.

Author Contributions: For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used “Conceptualization, JMM and RHB; methodology, RHB, ACO, and JPPN; software, ACO, RHB, MGVS and LDR.; validation, KENC, GMGG, and ASG; formal analysis, KENC, MGVS and LDR; investigation, RHB, ACO and JMM; resources, JMM, VHRA and RHB.; data curation, RHB, JMM, MGVS and LDR; writing—original draft preparation, ACO and RHB.; writing—review and editing, JMM, RHB and VHRA; visualization, JMM and RHB; supervision, JPPN and KENC; project administration, JMM and RHB; funding acquisition, JMM, RHB and VHRA. All authors have read and agreed to the published version of the manuscript.” Please turn to the CRediT taxonomy for the term explanation. Authorship must be limited to those who have contributed substantially to the work reported.

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