microRNA analysis of *Taenia crassiceps* cysticerci under praziquantel treatment and genome-wide identification of *Taenia solium* miRNAs

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Abstract

MicroRNAs (miRNAs) are small non-coding RNAs that have emerged as important regulators of gene expression and exert critical functions in development and disease. In spite of the increased interest in miRNAs from helminth parasites, no information is available on miRNAs from *Taenia solium*, the causative agent of cysticercosis, a neglected disease affecting millions of persons worldwide. Here we performed a comprehensive analysis of miRNAs from *Taenia crassiceps*, a laboratory model for *T. solium* studies and also identified miRNAs in the *T. solium* genome. Moreover, we analysed the effect of praziquantel, one of the two main drugs used for cysticercosis treatment, on the

miRNA expression profile of *T. crassiceps* cysticerci. Using small RNA-seq and two independent algorithms for miRNA prediction as well as Northern blot validation, we found transcriptional evidence of 39 miRNA loci in *T. crassiceps*. Since miRNAs were mapped to the *T. solium* genome, these miRNAs are considered common to both parasites. The miRNA expression profile of *T. crassiceps* was biased to the same set of highly expressed miRNAs reported in other cestodes. We found a significant altered expression of miR-7b under praziquantel treatment. In addition, we searched for miRNAs predicted to target genes related to drug response. We performed a detailed target prediction for miR-7b and found genes related to drug action. We report an initial approach to study the effect of sub-lethal drug treatment on miRNA expression in a cestode parasite, which provides a platform for further studies of miRNA involvement in drug effects. The results of our work could be applied to drug development and provide basic knowledge of cysticercosis and other neglected helminth infections.

Keywords: microRNA; praziquantel*; Taenia solium; Taenia crassiceps;* cestodes; microRNA targets; small RNA-seq.

1. Introduction

Taenia crassiceps is a cestode parasite of wild and domestic animals. It is widely used as an experimental model for *Taenia solium*, the etiological agent of neurocysticercosis (NC), the most common helminthic disease of the nervous system in humans (Singh et al., 2013). This disease is one of the 18 Neglected

Tropical Diseases prioritised by the WHO (http://www.who.int/neglected_diseases/diseases/en/) and accounts for about 50,000 deaths per year (Román et al., 2000).

Although humans rarely serve as intermediate hosts for *T. crassiceps*, there have been reported cases involving the muscles or subcutaneous tissue, associated with underlying immunosuppression (Chermette R, Bussieras J, Marionneau J, Boyer E, Roubin C, 1995; Goesseringer et al., 2011; Heldwein et al., 2006). In contrast, intraocular infections and one reported case of cerebellar cysticercosis (Arocker-Mettinger E1, Huber-Spitzy V, Auer H, Grabner G, 1992; Ntoukas et al., 2013) have been reported in immunocompetent patients. The only two antiparasitic drugs commonly used for NC and *T. crassiceps* treatment are albendazole and praziquantel (PZQ) (Ntoukas et al., 2013). PZQ is also used for infections caused by trematodes and other cestodes, and resistance to the drug has been reported (Chai, 2013), highlighting the importance of understanding the mechanisms of drug action and searching for alternative therapeutic options.

microRNAs (miRNAs) are non-coding RNAs of 21-25 nucleotides in length, which down-regulate gene expression post-transcriptionally by binding with partial sequence complementarity, most commonly to the 3' untranslated region (UTR) of their target mRNAs (Ambros, 2004). Nucleotides 2-8 of the mature miRNAs, referred to as the seed sequence, are essential in determining binding specificity in animals (Bartel, 2009). Detailed information on miRNAs expressed by parasitic helminths has been reported (Britton et al., 2012; Cai et al., 2016; Winter et al., 2015), being miRNA sequences available at the miRBase database (Kozomara and Griffiths-Jones, 2014). While some miRNAs are

shared even among highly divergent species, suggesting conserved roles/functions throughout evolution, many species have unique miRNAs based on current data. Over the last 5 years, there has been an increasing interest in the identification and characterization of miRNAs from parasitic cestodes, such as the zoonotic parasites *Echinococcus granulosus sensu stricto* (s. s.)*, Echinococcus multilocularis* and *Echinococcus canadensis* (Bai et al., 2014; Cucher et al., 2015, 2011) and the laboratory model *Mesocestoides corti* (Basika et al., 2016). However, in the genus *Taenia*, the only information available about miRNAs was obtained from adult worms from *Taenia saginata* (Ai et al., 2012) and *Taenia multiceps* (Wu et al., 2013). At present there is no bioinformatic or experimental evidence of the miRNA repertoire in *T. solium* or *T. crassiceps*.

A number of recent studies have demonstrated that drug metabolising enzymes and transporters, as well as drug targets, can be post-transcriptionally regulated by miRNAs and, importantly, those changes in the expression level of many miRNAs can be induced by drug treatment (Meng et al., 2006). It was suggested that miRNAs could play a role in drug resistance also in parasitic helminths (Devaney et al., 2010). However, no further studies have addressed this issue in cestodes to date. Identifying the function of specific miRNAs in parasitic species by experimental approaches is not an easy task (Xia et al., 2009) since *in vitro* culture and RNAi systems have been successfully accomplished only for few species (Bai et al., 2014; Guidi et al., 2015; Mizukami et al., 2010; Wang et al., 2014). As a first step to experimental validation, bioinformatic prediction of miRNA targets has been performed for a number of helminth parasites (Bai et al., 2014; Fu et al., 2013; Huang et al., 2009; Ma et

al., 2016; Winter et al., 2015; Zhu et al., 2016). However, there is no information of miRNA targeted genes in the genus *Taenia*.

In this report, we aimed at identifying the miRNA repertoires of *T. crassiceps* and *T. solium* and analysing the expression profile of *T. crassiceps* cysticerci with and without PZQ treatment as well as predicting miRNA targeted genes.

2. Material and Methods

2.1. Source of parasite material

Three biological replicates of *T. crassiceps* cysticerci (HYG strain) were each obtained from the peritoneal cavity of experimentally infected female CF1 mice (6–8 weeks old). Mice were sacrificed by cervical dislocation. The cysticerci were removed from the peritoneal cavity and washed at room temperature with PBS 1X solution. For all experiments, we inspected the cysticerci under stereoscopic microscope and classified them in stage I (translucent, non-budding), II (translucent, with budding) and III (whole opaque cyst, final stage), according to adapted classification of (Vinaud et al., 2007). Only vital cysticerci classified as stage I and II were used for the experiments. Mice were housed at the animal facilities of Instituto de Investigaciones en Microbiología y Parasitología Médica (IMPaM), Facultad de Medicina, Universidad de Buenos Aires (UBA)-Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET), Buenos Aires, Argentina in a temperature-controlled light cycle room with food and water *ad libitum*.

Experiments involving the use of experimental animals were carried out according to approved protocols by the Comité Institucional para el Cuidado y Uso de Animales de Laboratorio (CICUAL), Facultad de Medicina, Universidad de Buenos Aires (protocol number CD N° 1229/2015)

2.2. Experimental design and parasite culture

Thirty cysticerci from each of the 3 biological replicates were cultured either in absence (control samples, CTR) or presence of praziquantel (PZQ samples). Six samples were analyzed in total. Culture conditions were as follows: DMEM (Dulbecco's Modified Eagle Medium, high glucose with L-glutamine) (Gibco) supplemented with levofloxacine 20 µg/ml and gentamicin 50 µg/ml, at 37 ºC for 24 h under 5% CO2. Praziquantel was added at sublethal dose as determined by previous titration assays. Briefly, groups of 30 cysticerci each (stage I and stage II) were maintained in DMEM culture medium at 37 ºC with 5% CO2 and exposed for 24 h to increasing PZQ concentrations (0.03, 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 µg/ml). As controls, 30 cysticerci of the same stages were maintained in parallel in DMEM with the addition of DMSO at the same final concentration as in the PZQ samples (100mM). After 24 h of exposure to the drug, vitality was determined. The sublethal dose found was 0.05 µg /ml, in accordance with a previous report (Vinaud et al., 2008). In Supplementary Fig. S1 cysticerci treated with the sublethal and lethal doses of PZQ diluted in DMSO (100 mM final concentration) are shown. For determination of vitality, three groups of 10 cysticerci each (stages I and II) from the peritoneal cavity of the same mouse of each replica were maintained in

parallel in DMEM and in DMEM+DMSO (100mM). The criteria to assess parasite vitality were absence of loss of vesicular fluid, paralysis, collapse and staining with Trypan blue (0.05%). Only parasite samples with 100% vitality were used for small RNA isolation and library construction. All the experiments were performed with three biological replicates.

2.3. Small RNA isolation

RNA enriched in small RNAs (<200 nt) was purified from 30 cysticerci per replicate, using the mirVana miRNA Isolation Kit (Ambion) according to the manufacturer instructions. RNA was then precipitated overnight at -20 ºC with 0.1 volumes of 3 M sodium acetate (pH 5.2), 2.5 volumes of ethanol and glycogen. RNA was centrifuged at 14,000 g for 30 min at 4ºC, washed in 75% ethanol, air dried at room temperature and resuspended in 20 µl of nuclease-free water. Samples were stored at -80 ºC until library construction. RNA concentration was determined using a Qubit Fluorometer (Invitrogen-USA) and RNA integrity was assessed in an Agilent 2100 Bioanalyzer small RNA chip (Agilent Technologies).

2.4. Library construction and small RNA-seq

For each sample type (CTR and PZQ), three libraries were constructed from three independent samples in order to obtained biological replicates, totalizing six libraries. The small RNA library construction was performed with a TruSeq small RNA Library Prep Kit and sequenced on a HiSeq 2500 System.

Library size selection was performed for all the samples in order to recover RNAs ranging from 18 nt to 40 nt in length. small RNA libraries and sequencing experiments were performed at Macrogen, Korea.

2.5. Assembly and annotation of *T. crassiceps* small RNA

The *T. solium* genome (Tsai et al., 2013) was used for *T. crassiceps* miRNA identification since a *T. crassiceps* genome is not available. This also allowed the identification of *T. solium* miRNA precursors. Genome data of *Taenia solium* is available at http://www.taeniasolium.unam.mx/taenia/ and the complete genome annotation is available at www.genedb.org. Additional flatworms rRNA sequences and DNA repetitive elements were downloaded from NCBI. *Echinococcus* spp. miRNA and hairpin (pre-miRNAs) sequences, as well as metazoan mature miRNAs, were obtained from miRBase 21.0 and from previous studies for *Echinococcus* spp. (Cucher et al., 2015; Macchiaroli et al., 2015) and *M. corti* (Basika et al., 2016) as reported by our group. All the annotated sequences, together with the novel miRNA precursor sequences identified in this study, were used to construct an in-house database for small RNA library data classification available at http://www.bmhid.org/downloads/.

2.6. Bioinformatics analysis of Taenia crassiceps small RNAs

Illumina raw sequence reads produced by deep sequencing were pre-processed and collapsed as described previously (Cucher et al., 2015; Macchiaroli et al., 2015). To classify all small RNA library sequences the

processed reads were first mapped to the *T. solium* genome with Bowtie (version 1.1.0) with the option –v 2 that reports read mapping with up to two mismatches. All mapped reads were then analysed by BLASTN (e-value 0.01) against an in-house database available at http://www.bmhid.org/downloads/**,** which included all miRNAs identified in this study and were classified into miRNAs (see next section), rRNA, tRNA, CDS and others. Lastly, a length distribution analysis of the total mapped DNA reads and the total miRNA reads was performed.

2.7. miRNA identification

To identify previously reported and novel miRNAs from the small RNA libraries, the mirDeep2 software package was used as previously reported in (Cucher et al., 2015; Macchiaroli et al., 2015) but using instead *T. solium* as the reference genome instead, since *T. solium* is phylogenetically closer to *T. crassiceps* than either *E. multilocularis* or *E. granulosus*. All metazoan mature miRNAs and hairpins including the previously reported *Echinococcus* spp. sequences by (Cucher et al., 2015; Macchiaroli et al., 2015) were used as input. The initial miRDeep2 output list of candidate miRNAs of each library was manually curated to generate a final high confidence set of miRNAs retaining only candidate novel precursors with i) miRDeep2 score ≥ 4, ii) significant randfold p-value < 0.05, iii) mature reads in all the libraries (PZQ treated and control) and iv) presence of star strand. The secondary structures of putative precursors, with minimum free energy less than -17 kcal/mol and with a mature miRNA located in the stem, were predicted using the RNAfold software (Gruber

et al., 2008). The candidate novel precursor sequences were then analyzed using BLASTN (e-value 0.01) against sets of rRNAs, tRNAs, CDS, lncRNAs and repeats from the in-house database. Predictions that overlapped with these categories were removed. The final set of candidate *T. crassiceps* hairpins was searched against *T. solium* genome using BLAST to obtain the corresponding hairpin precursors. Additionally, we used an independent approach based on a deep architecture of self-organizing maps (SOMs), called miRNA-SOM (Kamenetzky et al., 2016) in order to confirm miRNA predictions and identify further miRNAs. Sequences with a minimum free energy threshold of -20 kcal/mol and single-loop folded sequences were selected according to the miRNA biogenesis model (Bartel, 2004) and then the best candidates were sequentially filtered in the SOM layers (Kamenetzky et al., 2016) and (Stegmayer et al., 2016).

2.8. miRNA abundance analysis

For analysis of miRNA abundance, read counts of each individual miRNA were normalised to the total number of mature miRNA read counts in each sample. Differential expression analysis of miRNAs between PZQ treated and untreated cysticerci was performed with DESeq software (Anders et al., 2010). This approach has been used in other miRNA studies (Dhahbi et al., 2011; Tonge et al., 2013). miRNAs expressed in both conditions that showed $-1 \geq \log2$ fold change ≥ 1 and adjusted *p* value adjusted < 0.05 were considered differentially expressed.

2.9. Experimental validation by Northern blot

Experimental validation of selected sequences was performed by Northern blot as described previously (Cucher et al., 2011) except that 6µg, 4µg and 2µg of small RNAs from control *T. crassiceps* cysticerci were used. The DNA probes (bantam: 5´-TCA GCT GTA ATC GCG ATC TCA-3´; let-7: 5´-AGA CAT TCG AAA CAC TAC CTC A-3´; miR-10: 5´CAA ACT CGG GTC TAC AGG GTG-3´; miR-71: 5´-TCT CAC TAC CAT CGT CTT TCA-3´; miR-4989: 5´-CTC AGA TAG TTG GTG CAT TT-3´; miR-277a: 5´CGG GCC AGA AAA TGC ATT TA-3´) were complementary to the putative *T. crassiceps* miRNAs and 5' end-labelled with γ^{32}P ATP (Perkin Elmer, NEN, USA). The probes were purified from the unincorporated label with Illustra microspin G-25 columns (GE Health Care). Before being use, the membrane was rinsed in 5x SSC (0.75 M NaCl, 75 mM sodium citrate), prehybridised for at least 90 min at 37 ºC in Ultrahyb-Oligo Hybridisation Buffer (Ambion) and incubated with the probe in the same buffer at 37 ºC overnight. After hybridisation, the membranes were washed twice in 2x SSC, 0.5% SDS at 32 ºC for 10 min and once at room temperature. Hybridisation bands were detected using a Typhoon Trio (Amersham Biosciences).

2.10. In silico prediction of miRNA targets

The miRanda algorithm (v3.3a) (Enright et al., 2003) was used to perform an independent prediction of miRNA target sites in *T. solium*. The parameters used were: i) strict seed pairing, ii) score threshold: 140, iii) energy threshold:

-17 kcal/mol, iv) gap open penalty: -9; v) gap extend penalty: -4; vi) scaling parameter: 4. Three hundred nucleotides were extracted downstream from the stop codon of *T. solium* genes using custom scripts. These sequences together with mature miRNAs identified in this study were used as input for miRNA prediction.

3. Results

3.1. miRNAs are abundantly expressed by T. crassiceps cysticerci

We obtained between 27 and 38 million raw reads per sample and observed 47.5 -71.5% mapping to the *T. solium* genome (Supplementary Table S1).

We found that the most abundant category for all the samples were miRNAs, accounting for 83% of mapped reads in both control and PZQ-treated cysticerci (Fig. 1, A-B). Length distribution analysis revealed one peak at 21 nt, compatible with miRNA length. No peak compatible with piRNAs (~ 30 nt) was observed in any sample, which suggests that piRNAs are not expressed in the cysticercus stage of *T. crassiceps*.

3.2. Taenia crassiceps and T. solium miRNA repertoire

We provided experimental evidence of *T. crassiceps* miRNA expression and identified miRNA precursors in the *T. solium* genome, this being the first time that a comprehensive repertoire of these small regulatory RNAs is

described in *T. crassiceps*. This is also the first report of a genome-wide identification of *Taenia solium* miRNAs. We obtained a final high confidence set of 41 miRNAs (Table 1), all of them conserved with other organisms (Macchiaroli et al., 2015). The identified miRNAs were grouped in 30 families according to their seed sequence. By using miRNA-SOM (Kamenetzky et al., 2016), we identified 3 additional miRNA precursors: pre-mir-7b, pre-mir-96 and pre-mir-3479b, conserved in *E. canadensis* (Cucher et al., 2015; Macchiaroli et al., 2015) that were not detected by miRDeep2. In addition, miRNA-SOM allowed us to confirm 2 miRNA precursors: pre-mir-new-1a and pre-mir-new-1b that were only described before in *E. granulosus* s.s. G1 genotype (Bai et al., 2014). Since a dominant mature miRNA can be processed either from the 5' or 3' arm of the corresponding pre-miRNA, we considered that both arms of the same hairpin produced two mature miRNAs when the number of read counts of the minor product represented ≥ 30% of the read counts from the major product originating from the opposite arm (Basika et al., 2016; Cucher et al., 2015). By doing this, we observed that pre-mir-31 and pre-mir-new-1a showed expression from both arms in both conditions (Supplementary Table S2). The sequences of mature and star miRNAs, as well as the corresponding precursors are shown in Supplementary Table S3. It can be observed that the total number of *T. crassiceps* precursors is 39. However, since two of the precursors showed expression from both arms, the number of mature miRNAs is 41 (Table 1). Since all *T. crassiceps* identified miRNAs were mapped to the *T. solium* genome this study provides the first report of miRNA precursor sequences for this zoonotic parasite.

3.3. Conserved and novel miRNA clusters are present in the T. solium genome

We found one protostomian-conserved miRNA cluster (mir-71/2c/2b, Fig. 2A), one cestode conserved cluster (mir-277/4989, Fig. 2B) (Basika et al., 2016), one family Taeniidae specific cluster (mir-new-1a/new-1b, Fig. 2C), one possible metazoan-conserved miRNA cluster (mir-1/133) and one miRNA cluster that was not reported before, which would be specific for *T. solium* formed by mir-7b and mir-3479a separated by a 5674 nt region (Supplementary Table S4). Cluster miR-new-1a/new-1b, comprises a 249-nt region and was only reported previously for *E. granulosus* s. s. G1 (Bai et al, 2014). According to previous reports, platyhelminth miRNA clusters size up to 500 bp (Cucher et al., 2015; Sasidharan et al., 2013; Wang et al., 2010). Hence, for cluster miR-7b/3479a as well as for miR-1/133, which is separated by 11618 nt, further assays should be performed to determine their co-transcription as a polycistronic unit. We analysed the small RNA-seq expression profile of members of the miR-71/2b/2c cluster and found, in control as well as in PZQ-treated cysticerci, that miR-71 from cluster miR-71/2b/2c shows greater than ten-fold higher expression compared with miR-2b, suggesting that post-transcriptional regulation takes place. For the cluster miR-new-1a/new-1b, miR-new-1a showed two-fold higher expression than miR-new-1b, while for the cluster miR-277/4989 a similar expression level was found between both members of the same cluster. However, in PZQ- treated cysticerci miR-4989 expression is 1.5 fold higher than miR-277 (Table 1). Here, we also analysed, according to small RNA-seq data, the expression level of miRNAs organized in clusters that are very likely to share a promoter. We found, in control as well as

in PZQ-treated cysticerci, that miR-71 from cluster miR-71/2b/2c shows more than ten-fold higher expression compared with miR-2b (t-test, $p<0.05$), suggesting that post-transcriptional regulation takes place.

3.4. Taenia crassiceps miRNA expression is biased to the same set of miRNAs than in other cestodes

We have previously shown that RNA-seq is highly accurate for quantifying expression levels in cestode parasites (Macchiaroli et al., 2015). Hence, here we used RNA-seq data to analyse miRNA expression profiles (Table 1). The top five most abundant miRNAs expressed in *T. crassiceps* cysticerci, that together account for 90 or 91% of total miRNA expression, were miR-10-5p, let-7-5p, miR-71-5p, bantam-3p and either miR-61-3p (control cysticerci) or miR-4989-3p (PZQ-treated cysticerci) (Fig. 3). Interestingly, miR-10-5p was the most abundantly expressed miRNA in both sample types, accounting for about 60% of the total miRNA expression in each sample.

In order to perform a comparative miRNA expression analysis in cestode parasites we compared the average normalized proportion of *T. crassiceps* miRNAs with that previously reported in other cestodes, i.e. *E. multilocularis* and *E. canadensis* (Cucher et al., 2015) and *M.corti* (Basika et al., 2016) (Fig. 4). We found that a set of miRNAs comprised by miR-10-5p, let-7-5p, bantam-3p, miR-71-5p, miR-4989-3p, miR-61-3p and miR-9-5p is abundantly expressed in the larval stages of the four cestodes analysed, with miR-10-5p being most abundantly expressed. We also observed that miR-36, a miRNA with low expression in both species of *Echinococcus* and in *M. corti*, showed high

expression in *T. crassiceps*. It is important to consider that some of the highly expressed miRNAs in cestodes are absent in the host or highly divergent in sequence with host orthologs (Macchiaroli et al., 2015), highlighting their potential as drug and/or diagnosis targets.

3.5. Taenia crassiceps miRNAs and precursors can be detected by Northern blot

We analysed the expression of six miRNAs: miR-10, let-7, miR-71, bantam, miR-4989 and miR-277a by Northern blot. A hybridization signal characteristic of mature miRNAs (~22 nt) was observed for these six miRNAs (Fig. 5) and an additional band of ~70 nt was present for the three with higher number of reads: miR-10, miR-71 and let-7 (Fig. 5A, 5B and 5C). The expected sizes of precursor bands are 77 nt (pre-mir-10), 61 nt (pre-mir-71) and 57 nt (pre-mir-let-7), this being the first time that a pre-miRNA is detected in a cestode parasite. It is interesting to note that miR-10 showed two bands corresponding to mature miRNAs (Fig. 5A). This is in accordance with the fact that similar sequences with slight length differences were present as isomiRs in the RNA-seq data (Supplementary Table S5).

3.6. Taenia crassiceps miRNA expression under PZQ treatment

We performed a correlation analysis between three independent biological replicates from each sample type, which indicated high reproducibility (correlation coefficient > 0.92) (Supplementary Fig. S2), thus allowing

differential expression analysis. We found that miR-7b-5p was ~six-fold up regulated in PZQ-treated versus control cysticerci ($p < 0.01$). Additionally, miR-7a-5p, miR-4989-3p, miR-31-3p also showed altered expression in treated parasites, although without statistical significance. The expression of the remaining miRNAs underwent no change with the PZQ dose and time of exposure used in this study (Supplementary Table S6and Supplementary Fig. 3).

3.7. Prediction of miRNA functional roles in Taenia

In order to analyse the possible roles of miRNAs in *Taenia*, we conducted a bioinformatic target prediction in the *T. solium* genome for *T. crassiceps* mature miRNAs. The results of the genome-wide target prediction of all the *T. solium/T. crassiceps* identified miRNAs are shown in Supplementary Table S7. It was found that 2471 protein coding genes were predicted targets of miRNAs, representing 22% of total coding genes. The miRNA that showed the highest number of targets was miR-71. Since we found that miR-10 was highly expressed in *T. crassiceps*, representing more than half of all miRNAs expressed (Fig. 3), a common feature of other cestodes (Fig. 4), we performed a detailed target analysis for this miRNA. Among miR-10 targets in *T. solium* we found two hox genes (Supplementary Table S8), both belonging to the ANTP class, one of them from the Meis family and the other from the Hox9-13 family (Tsai et al., 2013). Since miR-10 was shown to be located in hox gene clusters in vertebrates and *Drosophila* (Mansfield and McGlinn, 2012), we searched for adjacent genes to the mir-10 locus in the *T. solium* genome. Interestingly, we

found two homeobox-containing genes from the ANTP class upstream of the mir-10 locus: TsM_000864600 (Homeobox protein mab-5) and TsM_000522200 from Hox4 family (Tsai et al., 2013). Apart from the two homeobox-containing genes predicted to be targeted by miR-10, several genes related to regulation of transcription were found among the potential targets of this miRNA. Interestingly, a gene coding for a high mobility group protein, showed two target sites for miR-10. Besides control of gene transcription, other biological processes potentially regulated by miR-10 are carbohydrate metabolism, proteolysis, microtubule-based movement and calcium ion transport. We also made a detailed search for targets of miR-7b, the miRNA that showed increased expression upon drug treatment (Supplementary Table S9). Targets of miR-7b are involved in several pathways such as amino acid and nucleotide metabolism, vesicular transport, signaling pathways, cell adhesion, cell growth, cell death and interaction with neuroactive ligands. Also, a gene coding for calponin, a calcium binding protein that inhibits myosin related to muscle activity, was found among the potential targets of this miRNA.

Other miRNAs showed differences in expression level, although without statistical significance. In particular, we focused on miR-31, which showed a decreased expression level in treated parasites. The predicted targets of this miRNA include ABC transporters (drug efflux transporters), thioredoxins (involved in drug metabolism) and voltage-dependent L-type calcium channel subunit alpha-1D, which is a probable target of PZQ (Greenberg, 2005). In addition, we found targets related to drug efflux, metabolism and action (Table 2). It would be interesting to experimentally validate target prediction results in order to confirm these bioinformatic predictions.

4. Discussion

In this study we report for the first time a high confidence miRNA repertoire from *T. crassiceps* and *T. solium* zoonotic parasites and show that miRNAs account for most small RNA expression in *T. crassiceps* cysticerci. Since our miRNA identification strategy required the matching of *T. crassiceps* small RNA sequences to the *T. solium* genome, the identified miRNAs are considered common to both species, as it was previously considered for other helminthic parasites (Cucher et al., 2011; Winter et al., 2012). Hence, in this study, by combining experimental data from the model parasite *T. crassiceps* with genomic information from *T. solium*, we were able to identify miRNAs and their precursors in the genome of the aetiological agent of NC, a neglected disease that affects millions people worldwide (Asnis et al., 2009). The percentage of miRNAs in *T. crassiceps* cysticerci reaches 83% of the total small RNA expression, suggesting important functions of this type of RNA in the biology of taenias. With respect to other small regulatory RNAs, piRNAs were not detected. This result is in agreement with the absence of piRNAs in other platyhelminths (Macchiaroli et al., 2015; Skinner et al., 2014) and the absence of PIWI protein in the *Echinococcus* and *T. solium* genomes (Tsai et al., 2013; Zheng et al., 2013).

Here, we identified and validated miRNAs by Northern blot experiments. This validation is especially important in the case that genome data from other species is used, as in this case (Rosenzvit et al., 2013). Additionally, we experimentally detected pre-miRNAs for the first time in cestodes. From the 6

analysed miRNAs, it was possible to detect a pre-miRNA band for the most abundant mature miRNAs (see Fig. 5), adding confidence to the miRNA identification procedure performed in this work.

In this study, miRNA identification from RNA-seq data was based on two different miRNA prediction programs: miRNA-SOM (Kamenetzky et al., 2016) and miRDeep2 (Friedlander et al., 2012). The machine-learning based algorithm miRNA-SOM, which uses only genomic data, confirmed five miRNAs, including miRNAs specific for the Taeniidae family and miRNAs with a particularly long hairpin, in the draft genome of *T. solium*.

The *T. solium* and *T. crassiceps* miRNA catalog includes 41 conserved miRNAs grouped in 28 families. The number of conserved miRNA families is similar to that of *E. canadensis* (Macchiaroli et al., 2015) and *M corti* (Basika et al., 2016) (28 conserved miRNA families), providing further evidence for the loss of conserved miRNA families in cestodes as proposed by (Fromm et al., 2013). The miRNA catalog includes miR-10293-3p, a miRNA reported for the first time in *Echinococcus* (Cucher et al., 2015) and later in *M. corti* (Basika et al., 2016), thus confirming the presence of this novel miRNA in other cestodes. In addition, we also report two miRNA precursors (pre-mir-new-1a and pre-mir-new-1b) arranged in a cluster that were only reported before for *E. granulosus* s. s. G1 genotype (Bai et al., 2014). The first of these precursors shows expression from both arms in *T. crassiceps*, unlike *E. granulosus* s. s. G1 genotype that only expressed the 3p arm. This mature miRNA was named egr-new-22 in that report (Bai et al., 2014). The novel potential cluster found in this work is formed by the conserved miRNAs miR-7b and miR-3479a. Although both miRNAs are present in *Echinococcus* or *M. corti*, they are not clustered, highlighting

differences in the genome organization of miRNA precursors among cestodes. Until now, the importance of these differences in genomic arrangement is unknown but could potentially influence the expression of the corresponding mature miRNAs. With respect to cluster miR-71/2c/2b it was found only once in the genome of *T. solium*, like in other cestodes analysed to date (Cucher et al., 2015; Macchiaroli et al., 2015, Basika et al., 2016). This differs from other flatworms such as the monogean *Gyrodactylus salaris* and the trematode *Schistosoma mansoni* for which at least two miR-71/2 clusters were found (Fromm et al., 2013). The presence of only one miR-71/miR-2 cluster seems to be a common feature of cestode genomes. The uneven expression found among miRNAs of this cluster was also observed in *Echinococcus* spp (Cucher et al., 2015; Macchiaroli et al., 2015) and *M. corti* (Basika et al., 2016). With respect to the novel-miRNA cluster miR-new1a/new1b and the miR-277/4989 cluster, a similar expression level was found between members of the same cluster, suggesting that the main control of mature miRNA expression is, in these cases, at the transcriptional level, unlike what was observed in the *Echinococcus* miR-277/4989 cluster. These findings highlight differences in miRNA expression regulation among parasites. However, in PZQ-treated *T. crassiceps* cysticerci miR-4989 expression is 1.5 fold higher than miR-277 (t-test, $p>0.05$), suggesting that PZQ treatment induces post-transcriptional regulation of miRNA expression.

We also analysed the expression profile of control *T. crassiceps* cysticerci and found that miRNA expression is highly biased to a few miRNAs: miR-10, let-7, miR-71, bantam and miR-61. These five miRNAs account for ~90% miRNA expression. Coincidentally, in other reports of small RNAs from

cestodes that used the same methodology for miRNA discovery, miR-10, let-7, miR-71 and bantam were the most highly expressed, suggesting important functions in cestode biology (Cucher et al., 2015; Macchiaroli et al., 2015; Basika et al., 2016). Among these miRNAs, miR-10 is the most expressed. This miRNA is highly conserved across metazoan organisms and is implicated in *Hox* gene regulation, embryonic development, and cancer (Cai et al., 2011; Giusti et al., 2016; Zhi et al., 2015). It is interesting to note that among predicted miR-10 targets in *T. solium* we found two homeobox genes (Supp Table S7), both belonging to the ANTP class and also found pre-mir-10 located adjacent to other 2 hox genes. This result was expected since miR-10 Hox gene targeting and genomic localization in a hox cluster is a common feature of many bilaterians (Mansfield and McGlinn, 2012). It would be interesting to analyze miR-10 expression profile in different developmental stages in order to get insights on the molecular basis of development of these parasites.

Here we investigated the miRNA expression profile of *T. crassiceps* cysticerci incubated during 24 h with sublethal doses of PZQ, one of the main antiparasitic agents used for cysticercosis and taeniasis treatments, being this the first time that the effect of a drug in miRNA expression is analysed in cestodes. Our results showed that, in the conditions here assayed, the overall miRNA profile remained unchanged under PZQ treatment, except for miR-7b that showed a 6-fold enhanced expression. One of the predicted miR-7b targets was calponin, a calcium binding protein that inhibits myosin. This may be related to the expected alteration of intracellular calcium concentration produced by PZQ, a drug binding and inhibiting voltage-gated calcium channels, a key molecule for the regulation of calcium level inside the cell. Also,

we found that other targets of miR-7b were involved in several pathways such as amino acid and nucleotide metabolism, vesicular transport, signaling pathways, cell adhesion, cell growth, cell death and interaction with neuroactive ligands, suggesting the importance of this miRNA in parasite biology.

Other incubation conditions, such as exposition to the drug for longer periods, should be assayed in order to determine if this miRNA shows a significant altered expression profile in PZQ-exposed cysticerci. Although more studies will be necessary to understand the response of miRNAs to drug treatment and the influence that these regulators may have on drug action and/or drug resistance, the results here obtained pave the way for such type of analyses.

In conclusion, the present study represents the first characterization of *T. solium* and *T. crassiceps* miRNA repertoire. For the first time, the effect of drug (PZQ) treatment on miRNA expression was studied in a cestode parasite, providing the first step in such type of analysis, which may be crucial to understand drug response in these parasites. In addition, we identified highly expressed miRNAs among cestodes and predicted their target mRNAs finding important roles in parasite development and survival in the host. Given the importance of miRNAs in cellular processes such as development and proliferation, the information here provided constitutes a platform for miRNA functional studies and potential applications to the control of cestode parasites that are the cause of complex and neglected diseases.

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Figure legends

Fig. 1. Small RNA library composition and length profile of *Taenia crassiceps* cysticerci sequencing data. Classification of the RNA species obtained in the datasets from control (A) and praziquantel (PZQ) treated *T. crassiceps* cysticerci (B). Reads mapping to lncRNAs and repeats, and reads with no formal annotation (Unknown) are not represented due to their low relative abundance (<0.0001%). Results are shown as average percentage (±S.D).

Fig. 2. Three clusters of *Taenia solium* miRNAs. The secondary structure of *T. solium* clusters containing miR-71, miR-2b and miR-2c (A); miR-277 and miR-4989 (B) and miR-new-1a and miR-new-1b (C) were predicted by mfold.

Fig. 3. Top five more abundant miRNAs in *Taenia crassiceps* cysticerci. The average proportion of the top five most abundant miRNA reads normalized to the total number of mature miRNAs in each library is shown for control (A) and praziquantel treated (B) cysticerci.

Fig. 4. Comparative expression profile of the full miRNA repertoire of cestodes. Expression data corresponds to *Echinococcus multilocularis* (Em) and *Echinococcus canadensis* G7 (Ec) metacestodes (Cucher et al, 2015), control *Taenia crassiceps* cysticerci (Tc) (this work) and *Mesocestoides corti* tetratyridia (Mc) (Basika et al, 2016). Heatmap of log2-transformed normalized miRNA reads organized by their transcriptional abundance. miRNA expression is displayed using a color key where blue corresponds to low and red to high numbers of miRNA normalized reads.

Fig. 5. Validation of miRNA expression by Northern blot. Small RNA (<200 nt) from a mixture of control *T. crassiceps* cysticerci were resolved by 15% denaturating PAGE and transferred to nylon membranes. Ethidium bromide staining of the gels shows that equivalent amounts of RNA were loaded. The membranes were probed with ^{32}P –labelled oligonucleotides for detection of

miR-10 (A), let-7 (B), miR-71(C), bantam (D), miR-4989 (E) and miR-277 (F) . rpm: average reads per million for each miRNA.

Supplementary Fig. S1. Effect of different doses of praziquantel in *Taenia crassiceps* cysticerci stained with Trypan blue (0.05%). Cysticerci were treated with 0.05 µg/ml (sublethal dose) (A) and 0.20 µg/ml of praziquantel (B) for 24 hours, incubated in Trypan blue (0.05%) and washed with PBS 1x.

Supplementary Figure S2. Correlation analysis between independent biological replicates from control (Tc) and praziquantel treated (TcPZQ) *Taenia crassiceps* cysticerci samples. Each data point represents one miRNA. Pearson's correlation coefficient is shown in each plot.

Supplementary Figure S3. Comparative expression profile of control (Tc) and praziquantel treated (TcPZQ) *Taenia crassiceps* cysticerci. Heatmap of log2-transformed normalized miRNA reads organized by their transcriptional abundance. miRNA expression is displayed using a color key where blue corresponds to low and red to high numbers of miRNA normalized reads.