International Journal for Parasitology xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

Cestode parasites release extracellular vesicles with microRNAs and immunodiagnostic protein cargo

María Eugenia Ancarola [a], Antonio Marcilla [b,c], Michaela Herz [d], Natalia Macchiaroli [a], Matías Pérez [a], Sebastián Asurmendi [e], Klaus Brehm [d], Carolina Poncini [a], Mara Rosenzvit [a], Marcela Cucher [a,*]

[a] Instituto de Investigaciones en Microbiología y Parasitología Médica (IMPaM, UBA-CONICET), Facultad de Medicina, Universidad de Buenos Aires (UBA), Paraguay 2155, Piso 13, Buenos Aires, Argentina
[b] Área de Parasitología, Departamento de Farmacia y Tecnología Farmacéutica y Parasitología, Universitat de València, Burjassot, Valencia, Spain
[c] Joint Research Unit on Endocrinology, Nutrition and Clinical Dietetics, Health Research Institute-La Fe, Universitat de València, 46026 Valencia, Spain
[d] University of Würzburg, Institute of Hygiene and Microbiology, Josef-Schneider-Strasse 2, D-97080 Würzburg, Germany
[e] Instituto de Biotecnología, CICVyA-INTA, Dr. N. Repetto y Los Reseros s/n, 1686 Hurlingham, Buenos Aires, Argentina

ARTICLE INFO

Article history:
Received 30 December 2016
Received in revised form 21 April 2017
Accepted 1 May 2017
Available online xxxx

Keywords:
Cestode
Platyhelminth
microRNA
Extracellular vesicle
Secretion
Taenia
Mesocestoides corti
Echinococcus

ABSTRACT

Intercellular communication is crucial in multiple aspects of cell biology. This interaction can be mediated by several mechanisms including extracellular vesicle (EV) transfer. EV secretion by parasites has been reported in protozoans, trematodes and nematodes. Here we report that this mechanism is present in three different species of cestodes, *Taenia crassiceps*, *Mesocestoides corti* and *Echinococcus multilocularis*. To confirm this we determined, in vitro, the presence of EVs in culture supernatants by transmission electron microscopy. Interestingly, while *T. crassiceps* and *M. corti* metacestodes secrete membranous structures into the culture media, similar vesicles were observed in the interface of the germinal and laminated layers of *E. multilocularis* metacestodes and were hardly detected in culture supernatants. We then determined the protein cargo in the EV-enriched secreted fractions of *T. crassiceps* and *M. corti* conditioned media by LC-MS/MS. Among the identified proteins, eukaryotic vesicle-enriched proteins were identified as expected, but also proteins used for cestode disease diagnosis, proteins related to neurotransmission, lipid binding proteins as well as host immunoglobulins and complement factors. Finally, we confirmed by capillary electrophoresis the presence of intravesicular RNA for both parasites and detected microRNAs by reverse transcription-PCR. This is the first report of EV secretion in cestode parasites and of an RNA secretion mechanism. These findings will provide valuable data not only for basic cestode biology but also for the rational search for new diagnostic targets.

1. Introduction

Intercellular communication is crucial in multiple aspects of cell biology, from the proper development, growth and maintenance of single cell organism populations to the correct morphogenesis of multicellular organisms. This interaction is mediated by different mechanisms that may involve cell–cell contact, secreted soluble factors or extracellular vesicle (EV) transfer. The latter has been only recently acknowledged as an active intercellular transfer mechanism of proteins, nucleic acids and lipids instead of only being a process for disposal of unnecessary cell contents (Colombo et al., 2014). Interaction mechanisms are also important in cross-species communication, e.g. host-parasite interplay. In such a case, signalling between organisms is fundamental for the establishment, persistence and outcome of the corresponding parasitic disease. Hence, the identification and characterisation of these mechanisms provide valuable information to counteract such processes.

Among infectious diseases, the zoonoses produced by cestode parasite infections are associated with poverty and poor hygiene practices, particularly in livestock-raising communities, and cause debilitating chronic diseases which affect humans as well as domestic and wild mammals worldwide. In particular, echinococcosis and cysticercosis, caused by the metacestode stages of *Echinococcus* spp. and *Taenia solium*, respectively, are among the 17 most severe neglected tropical diseases in humans prioritized by the World Health Organization (http://www.who.int/neglected_diseases/diseases/en/).

Recently, it has been reported that protozoan as well as trematode and nematode parasites secrete EVs in vitro (Geiger et al., 2010; Silverman et al., 2010; Marcilla et al., 2012; Regev-Rudzki

* Corresponding author.
E-mail address: marcecucher@gmail.com (M. Cucher).

http://dx.doi.org/10.1016/j.ijpara.2017.05.003

et al., 2013; Bernal et al., 2014; Buck et al., 2014; Chaiyadet et al., 2015; Hansen et al., 2015; Nowacki et al., 2015; Wang et al., 2015; Zamanian et al., 2015; Tzelos et al., 2016). The term EV groups several types of vesicles among which microvesicles and exosomes are the most thoroughly characterised. They can be distinguished by size and morphology, as well as protein and lipid composition (Colombo et al., 2014). The protein content of EVs may reflect their biogenesis since, for instance, exosomes have an endocytic origin and hence display proteins involved in endocytosis and endosome formation, while microvesicles bud from the plasma membrane (Colombo et al., 2014).

Regarding the RNA content of EVs, they were found to carry both mRNAs and small RNAs (Valadi et al., 2007; Crescitelli et al., 2013). Among the latter, the presence of microRNAs (miRNAs) was confirmed in helminth EVs (Bernal et al., 2014; Buck et al., 2014; Fromm et al., 2015; Hansen et al., 2015; Nowacki et al., 2015; Zamanian et al., 2015). miRNAs are small non-coding RNAs that down-regulate their target gene products and have been shown to be actively secreted not only within EVs but also bound to proteins in mammalian models (Valadi et al., 2007; Vickers et al., 2011; Arroyo et al., 2011). Interestingly, the in vitro down-regulation of target host genes after the internalization of EVs from nematode parasites has been recently reported (Buck et al., 2014).

To date, information on EV secretion in cestode parasites is scarce and limited to ultrastructural studies (Ingold et al., 2000; Galán-Puchades et al., 2016). In the current study, we proposed to establish whether cestodes release EVs and, if so, to characterise their contents by analysing the metacestode stages of the model cestodes *Taenia crassiceps* and *Mesocestoides corti,* and the zoonotic species *Echinococcus multilocularis*.

2. Materials and methods

2.1. Parasite material

Taenia crassiceps and *M. corti* metacestodes were obtained from experimental infections maintained at the animal facilities of Instituto de Investigaciones en Microbiología y Parasitología Médica (IMPaM), Argentina. *Taenia crassiceps* cysticerci were maintained by serial i.p. passage in adult (6-week-old) CF1 female mice by inoculating 50 cysticerci. *Mesocestoides corti* tetrathiridia were maintained by serial i.p. passages in adult (3-month-old) BALB/c mice alternating every three passages with one in an adult (3-month-old) Wistar female rat by inoculation of 200 μl or 500 μl of tetrathiridia, respectively. Experiments involving the use of experimental animals were carried out according to approved protocols by the Institutional Committee for the Care and Correct Treatment of Laboratory Animals from the School of Medicine of University of Buenos Aires, Argentina, (protocols number CD N° 1127/2015 and 1229/2015).

Echinococcus multilocularis metacestodes from isolates H95, J2012, Ingrid, GH09 and MS10 were maintained by serial i.p. passage in *Meriones unguiculatus* at the animal facilities of the Institute of Hygiene and Microbiology, University of Würzburg, Germany, as previously described (Spiliotis and Brehm, 2009). Animal experiments were carried out in accordance with European and German regulations on the protection of animals (Tierschutzgesetz) and were approved by the government of Lower Franconia under permit no. 55.2-2531.01-61/13.

2.2. In vitro parasite culture

After recovery of *T. crassiceps* cysticerci and *M. corti* tetrathiridia from experimental animals, parasites were washed 3–5 times with sterile PBS and were then filtered through a 150 μm pore mesh, adding sterile PBS to remove murine cells or debris. Parasite viability was assessed before and after in vitro culture by Trypan blue staining at 0.002% final concentration. Only 100% viable parasites were used. Ten to 15 ml of *T. crassiceps* cysticerci were cultured in T75 flasks in an upright position with 125 ml of medium while 20–40 μl of *M. corti* tetrathiridia were incubated per well in 12-well plates. The medium used was DMEM with gentamicin (50 μg/ml) and levofloxacin (20 μg/ml) without serum. Parasites were incubated at 37 °C, 5% CO_2 for 1–4 days without medium change.

Axenic *E. multilocularis* metacestodes were obtained as previously described (Spiliotis and Brehm, 2009). Briefly, metacestodes were washed at least three times with sterile PBS. Collapsed and/or phenol red-stained metacestodes were removed. Approximately 30 ml of viable parasites were then transferred to a T175 culture flask with DMEM conditioned with rat Reuber hepatoma cells supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (0.1 g/l) and reducing agents, and were incubated at 37 °C in a nitrogen atmosphere (Spiliotis and Brehm, 2009). After 3 days, parasites were washed 3–5 times with sterile PBS, transferred to a new T175 flask with 120 ml of DMEM and reducing agents (without serum). Parasites were incubated at 37 °C in a nitrogen atmosphere for 4 days without medium change.

2.3. EV isolation

Culture media were collected and centrifuged according to Théry et al. (2006) but with modifications. Briefly, to obtain one EV sample, at least 55 ml of culture media from *T. crassiceps* and *M. corti* cultures were centrifuged for 20 min at 2,000*g* at 10 °C, and 30 min at 10,000*g* at 10 °C. The obtained supernatants were ultracentrifuged for 70 min at 100,000*g* at 4 °C in a Beckman Coulter Optima L-100 XP centrifuge using a fixed angle rotor, washed with PBS and ultracentrifuged again. In the case of *E. multilocularis* supernatants, to obtain one EV sample, 110–120 ml of culture medium were used and the ultracentrifugation step was performed in a Sorvall WX+ Ultracentrifuge (Thermo Scientific, Germany) with a TH-641 rotor. Pellets were resuspended in sterile PBS and used for transmission electron microscopy (TEM), proteomics or RNA content characterization.

2.4. Transmission electron microscopy (TEM)

The pellets obtained after the ultracentrifugation step were resuspended in PBS, fixed in Karnovsky's fixative (0.5% glutaraldehyde, 2.5% paraformaldehyde) and processed according to Marcilla et al. (2012) at the Service of Microscopy, Servicios Centrales de Soporte a la Investigación Experimental (SCSIE), Universitat de València, Spain. Parasites from each species were also fixed and analysed by TEM. In addition, ultracentrifugation pellets from *T. crassiceps* supernatants were negatively stained with 0.1% ammonium molybdate on a membrane acrylic–coated grid at Laboratorio Nacional de Investigación y Servicios de Microscopía Electrónica (LANAIS-MIE), School of Medicine, University of Buenos Aires.

2.5. Proteomic analysis

LC-MS/MS was performed on ultracentrifugation pellets according to Marcilla et al. (2012). The proteomic analysis was performed in the Proteomics facility of SCSIE, Universitat de València, which belongs to ProteoRed (PRB2- Instituto de Salud Carlos III, and supported by grant PT13/0001 of the PE I+D+i 2013-2016, funded by Instituto de Salud Carlos III and Fondo Europeo de Desarrollo Regional). The Paragon algorithm of ProteinPilot v 4.5 was used to search the National Center for Biotechnology Information (NCBI) complete Protein database with the following parameters: trypsin

Please cite this article in press as: Ancarola, M.E., et al. Cestode parasites release extracellular vesicles with microRNAs and immunodiagnostic protein cargo. Int. J. Parasitol. (2017), http://dx.doi.org/10.1016/j.ijpara.2017.05.003

specificity, cys-alkylation, no taxonomy restriction, and the search effort set to 'through' (Shilov et al., 2007). Reported results correspond to those proteins showing an unused score ≥1.3 (identified with confidence ≥96%), ≥2 distinct peptides having at least 95% confidence and cestode, rat or mouse protein sequence annotation.

2.6. Protein sequence analysis

After retrieval of the ProteinPilot results, assigned protein sequences were manually curated using BLASTp against the NCBI non-redundant protein sequences (nr) database. NCBI contains the complete genomes from model cestodes such as those from the genus *Echinococcus* and allows orthology analyses with the most characterised protein sequences uploaded in the database by using the SMART BLAST tool. Those proteins for which the reported sequence did not fully correspond to the annotated name in the database, e.g. due to lacking one or more relevant domains, were named only after the domain they display. Gene Ontology (GO) terms corresponding to the component category were assigned to the identified proteins. For this, Uniprot and GO identification terms (IDs) were retrieved from the Protein Information Resource site (http://pir.georgetown.edu/pirwww/search/idmapping.shtml). GO term descriptions were then downloaded from The European Bioinformatics Institute site (https://www.ebi.ac.uk/QuickGO/).

Proteins annotated under the terms "hypothetical protein", "expressed protein" or "conserved protein" were searched for domains in the domains database CDART (Geer et al., 2002) at the NCBI site, and re-annotated if necessary. Also, the same sort of sequences reported for EVs of trematodes (Marcilla et al., 2012; Bernal et al., 2014; Chaiyadet et al., 2015; Cwiklinski et al., 2015; Nowacki et al., 2015; Sotillo et al., 2016) were retrieved and likewise analysed for comparative purposes. When more than one protein sequence was reported for the same gene, the longest sequence was used for the analysis.

The signal peptide search was conducted on selected sequences with SignalP 4.1 using a sensitive d cut-off value (Petersen et al., 2011).

Phylogenetic analyses of selected sequences were conducted in MEGA7 (Kumar et al., 2016) by using the Maximum Likelihood method based on the JTT matrix-based model (Jones et al., 1992). A bootstrap consensus tree was inferred from 100 replicates. Branches corresponding to partitions reproduced in less than 50% of bootstrap replicates were collapsed. Sequences were retrieved from NCBI, Wormbase (ftp://ftp.wormbase.org/), Ensembl (ftp://ftp.ensembl.org/), Flybase (ftp://ftp.flybase.net/), GeneDB (ftp://ftp.sanger.ac.uk/), The *Gyrodactylus salaris* Genome Project (http://invitro.titan.uio.no/) and The *Taenia solium* Genome Project databases (ftp://bioinformatica.biomedicas.unam.mx/) (Maldonado et al., 2017).

2.7. RNA isolation

RNA from EVs and parasites was isolated with Trizol LS (Life Technologies, U.S.A.) and TriPure (Roche, Germany), respectively. The obtained aqueous phases were precipitated with 0.1 volumes of 3 M sodium acetate pH 5.2, 2.5 volumes of 100% (v/v) ethanol and 2 µl of glycogen (10 mg/ml) at −80 °C overnight followed by -20 °C for 1 day. RNA was centrifuged at 14,000*g* for 1 h at 10 °C. Pellets were air dried at 37 °C and resuspended in nuclease-free water. Cellular RNA integrity was analysed by gel electrophoresis. RNA concentration was determined using a Qubit Fluorometer (Invitrogen, U.S.A.).

To confirm the intravesicular location of the isolated RNA from the ultracentrifugation pellets, samples were treated prior to RNA isolation with proteinase K, RNAse A and/or SDS as previously reported (Montecalvo et al., 2012; Shelke et al., 2014) with modifications. Briefly, EVs were treated as follows: (i) control, (ii) proteinase K (0.5 µg/µl) 10 min at 37 °C, 10 min at 65 °C followed by incubation with RNAse A (0.04 µg/µl) 10 min at 37 °C and (iii) 0.5% SDS + proteinase K (0.5 µg/µl) 10 min at 37 °C, 10 min at 65 °C followed by incubation with RNAse A (0.04 µg/µl) 10 min at 37 °C. The corresponding RNA profiles were analysed by capillary electrophoresis in a Fragment Analyzer (Advanced Analytical Technologies, U.S.A.).

EV-depleted fractions (EV-free) were concentrated with 3 kDa Amicon Centrifuge Filter devices followed by two washes with PBS. RNA was isolated as described above. Two and three independent samples from *M. corti* and *T. crassiceps*, respectively, were analysed.

2.8. miRNA poly-A reverse transcription (RT)-PCR

miRNA cDNA synthesis was performed according to Macchiaroli et al. (2015) using 5 ng of input RNA. PCR was performed in a StepOne Plus cycler (Applied Biosystems, U.S.A.). The PCR mix consisted of 2.28 µl of 5X Hemo KlenTaq Buffer, 0.2 mM dNTPs, 0.1 µM of each primer, 0.32 µl of Hemo KlenTaq DNA Polymerase, 2X EVA Green, distilled water up to 18 µl and 2 µl of diluted cDNA. The cycling conditions were: 3 min at 95 °C, followed by 40 x (15 s at 95 °C, 32 s at 60 °C). For primer design, sequences of *M. corti* miRNAs were obtained from Basika et al. (2016). For *T. crassiceps* miRNAs, the sequences reported for *Taenia multiceps* were used (Wu et al., 2013). Primer sequences are shown in Supplementary Table S1. Two biological replicates from EVs from *T. crassiceps* and *M. corti* were used. Amplification products were assessed by gel electrophoresis.

2.9. Cestode miRNA binding site prediction on host genes

The Ensembl database (v.84) (Yates et al., 2016) was used to retrieve 3′ untranslated region (UTR) sequences for the protein coding genes from the *Mus musculus* GRCm38.p4 assembly. When more than one transcript was available for the same gene, only the longest isoform was considered. The miRanda algorithm (v3.3a) (Enright et al., 2003) was used to predict cestode miRNA target sites in *Mus musculus* 3′UTRs with the following parameters: (i) strict 5′ seed pairing; (ii) score threshold: 140; (iii) energy threshold: −20 kcal/mol; (iv) gap open penalty: −9; (v) gap extend penalty: −4; vi) scaling parameter: 4. *Echinococcus* miRNAs were used as input since they represent the most characterised dataset published to date for cestodes (Cucher et al., 2015; Macchiaroli et al., 2015). In addition, cestode miRNAs have a high degree of sequence identity (Basika et al., 2016).

Finally, functional annotation of the predicted targets was performed with the Panther classification system (http://pantherdb.org/) using the pathway classification (Mi et al., 2016).

3. Results

3.1. The metacestode stages of T. crassiceps, M. corti and E. multilocularis produce EVs

Currently, a generally accepted "gold standard" method to isolate and/or purify EVs is lacking (Lötvall et al., 2014). In order to determine whether cestode parasites secrete EVs, a methodology was chosen that allowed collection of a wide range of sizes of EVs. Hence, we performed the purification by differential centrifugation followed by ultracentrifugation; methodology that is chosen by more than 80% of the scientific community working in this field, mostly when large volumes from non-complex samples such as

Please cite this article in press as: Ancarola, M.E., et al. Cestode parasites release extracellular vesicles with microRNAs and immunodiagnostic protein cargo. Int. J. Parasitol. (2017), http://dx.doi.org/10.1016/j.ijpara.2017.05.003

cell culture media have to be processed (Gardiner et al., 2016). However, it is worth mentioning that other purification methods have been proposed in order to obtain purer samples, minimising the presence of contaminants such as soluble proteins (Gardiner et al., 2016).

In this way, the secretion of EV-like structures was confirmed by TEM for the metacestode stages of *T. crassiceps* (Fig. 1A and B) and *M. corti* (Fig. 2A and B) since round-shaped membrane-bound structures were isolated by ultracentrifugation from the axenic culture media of each parasite. Also, EVs with the same characteristics were detected in transit in the tegument of both parasites within structures compatible with multivesicular bodies, which are intermediate forms of the endocytic biogenesis pathway of exosomes (Figs. 1C–F, 2C–F). No vesicles budding from the tegument of these parasites were observed.

Interestingly, when analysing tissue sections of *E. multilocularis* metacestodes by TEM, EVs were observed in the interface between the laminated layer and the germinal layer (Fig. 3A–D). Few vesicles were observed in transit towards the exterior through the laminated layer and they were hardly detected in the supernatants of culture medium (data not shown). Structures compatible with multivesicular bodies and vesicles budding from the tegument were also observed (Fig. 3).

With respect to the diameters of the observed vesicles, sizes in the range of those reported for exosomes (<100 nm) and microvesicles (>100 nm) were detected for the three cestodes (Fig. 4). The most abundant population of EVs was not determined from these data since the sampling was quite different in *T. crassiceps* and *M. corti* with respect to *E. multilocularis*. As stated before, *E. multilocularis* EVs seem to be retained by the laminated layer while the other two parasites lack this structure. Hence, it is possible that *T. crassiceps* and *M. corti* secrete larger vesicles which were not detected by only analysing the ultracentrifugation pellets.

Since only *T. crassiceps* and *M. corti* secreted EVs outwardly, suggesting those represent an interaction mechanism with the host or other metacestodes, we proceeded with the characterisation of the protein and RNA content of these parasite EVs.

3.2. The EVs from T. crassiceps and M. corti contain typical EV-enriched proteins and immunodiagnostic antigens

Even though the protein content of the EVs depends on the cell of origin, there are some proteins which are regularly found. An exploratory analysis by liquid chromatography and tandem mass spectrometry, performed to identify the proteins associated with the EV-enriched fraction of *T. crassiceps*- and *M. corti*-conditioned

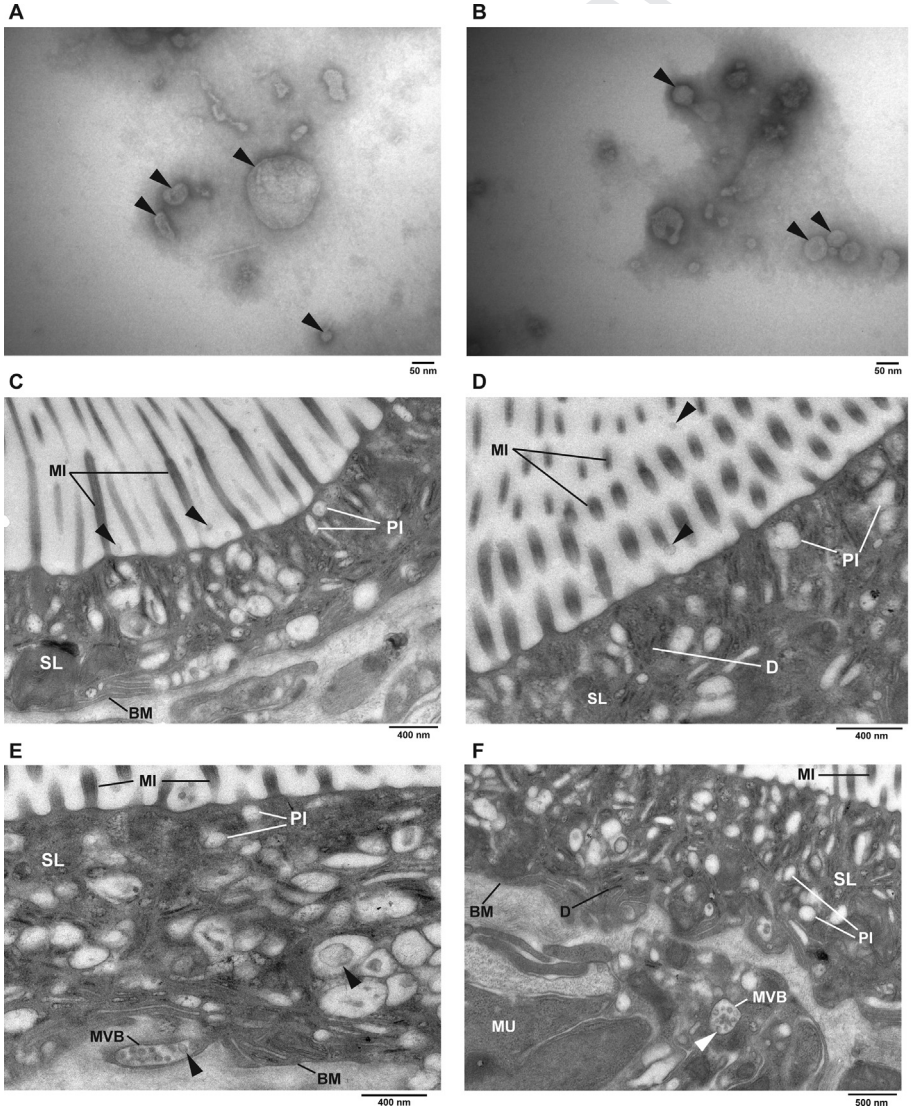

Fig. 1. *Taenia crassiceps* secretes extracellular vesicles (EVs). Transmission electron microscopy of culture supernatant (A, B), tegument surface (C, D) and tegument (E, F). Arrowheads indicate EVs. BM, basal membrane; D, dense secretory body; MI, microthrix; MU, muscle; MVB, multivesicular body; PI, pinosome; SL, surface layer.

Please cite this article in press as: Ancarola, M.E., et al. Cestode parasites release extracellular vesicles with microRNAs and immunodiagnostic protein cargo. Int. J. Parasitol. (2017), http://dx.doi.org/10.1016/j.ijpara.2017.05.003

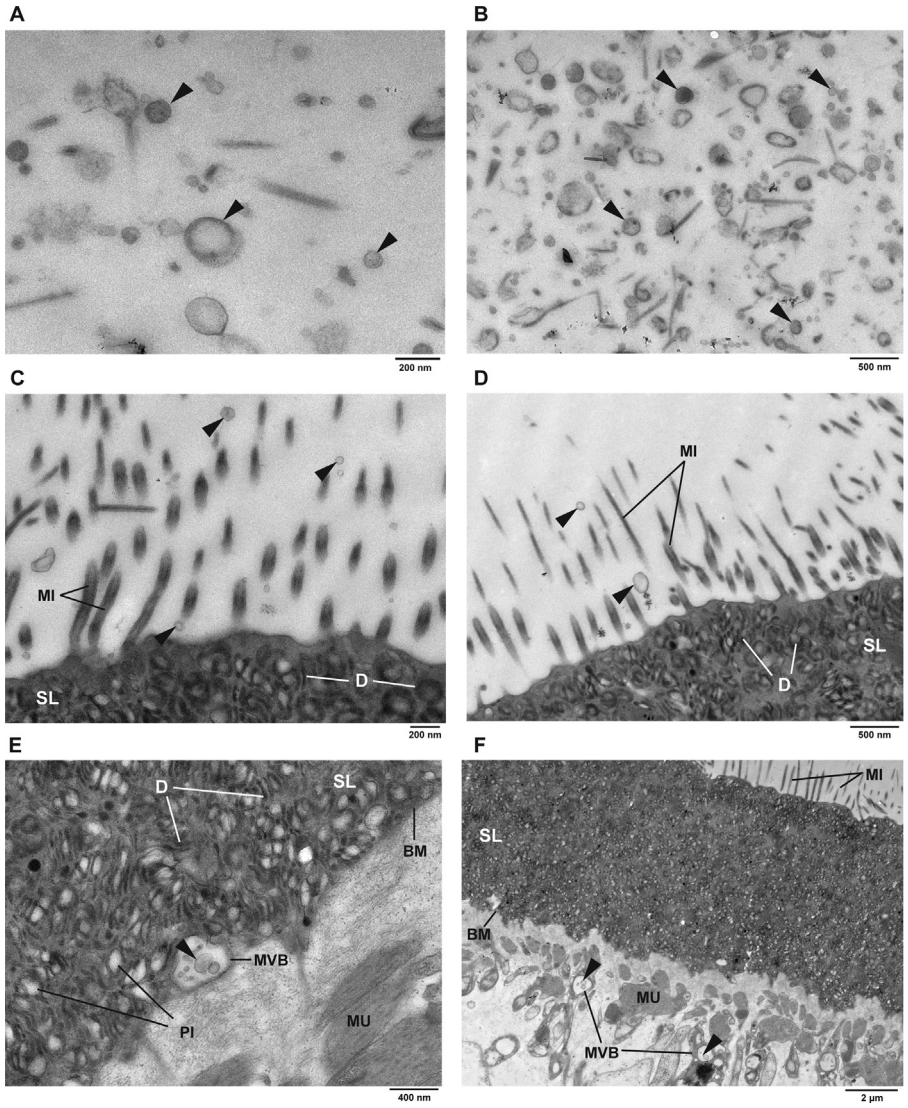

Fig. 2. *Mesocestoides corti* secretes extracellular vesicles (EVs). Transmission electron microscopy of culture supernatant (A, B), tegument surface (C, D) and tegument (E, F). Arrowheads indicate EVs. BM. basal membrane; D, dense secretory body; MI, microthrix; MU, muscle; MVB, multivesicular body; PI, pinosome; SL, surface layer.

media, showed the presence of proteins typically found in platyhelminth parasite EVs in both datasets (Marcilla et al., 2012; Bernal et al., 2014; Chaiyadet et al., 2015; Cwiklinski et al., 2015; Nowacki et al., 2015; Sotillo et al., 2016) and also in human and mouse EVs such as heat shock proteins, annexin, enolase, phosphoglycerate kinase, actin, tubulin, elongation factors and BROX. Table 1 summarizes the proteins identified in both parasites datasets. The main GO terms corresponding to the component category and associated with the detected proteins were "intracellular", "membrane" and "cytoplasm" (Supplementary Fig. S1), which group the EV-enriched proteins (Supplementary Tables S2 and S3).

Clathrin was identified in *T. crassiceps* EVs, which is in agreement with previous ultrastructural observations where clathrin-coated-like vesicles were detected in the tegument of cysticerci (Threadgold and Dunn, 1983). Also in the *T. crassiceps* dataset, proteins related to synaptic vesicle formation or neurotransmitter exocytosis (Munson, 2015) were detected, such as a BAR-domain containing protein (endophilin/p29), synaptic vesicle membrane protein VAT1, syntaxin-binding protein, synaptotagmin and syntaxin (Supplementary Table S2). In the *M. corti* dataset, two such proteins named synaptobrevin YKT6 and N-ethylmaleimide sensitive factor attachment were detected (Supplementary Table S3). It is worth mentioning that except for endophilin and N ethylmaleimide sensitive factor attachment, the remaining proteins were actually found in both datasets, however outside the cut-off values selected for protein identification. Endophilin has been also reported in *Fasciola hepatica* and *Opisthorchis viverrini* EVs (Chaiyadet et al., 2015; Cwiklinski et al., 2015), while synaptotagmin and syntaxin-binding protein have been detected in *F. hepatica* and *Schistosoma mansoni* vesicles, respectively (Cwiklinski et al., 2015; Nowacki et al., 2015).

Among distinctive proteins found in *T. crassiceps* and *M. corti* EVs, peptides belonging to proteins without formal annotation, i.e. annotated under the terms "expressed protein", "hypothetical protein" or "conserved protein", were identified (Supplementary Tables S2 and S3). One of these proteins was present in vesicles of both parasites, its amino acid sequence is highly conserved among cestodes (Supplementary Fig. 2A), has a UPF0047 domain with unknown function, no signal peptide sequence was predicted by SignalP 4.1 analysis and it has orthologs in other platyhelminths (both parasitic and free-living) such as *S. mansoni* and *Schmidtea mediterranea*, as well as in *Drosophila melanogaster* and the amphioxus *Branchiostoma floridae* (Supplementary Fig. 2A) (Maldonado et al., 2017). However, to date it was not found in

Please cite this article in press as: Ancarola, M.E., et al. Cestode parasites release extracellular vesicles with microRNAs and immunodiagnostic protein cargo. Int. J. Parasitol. (2017), http://dx.doi.org/10.1016/j.ijpara.2017.05.003

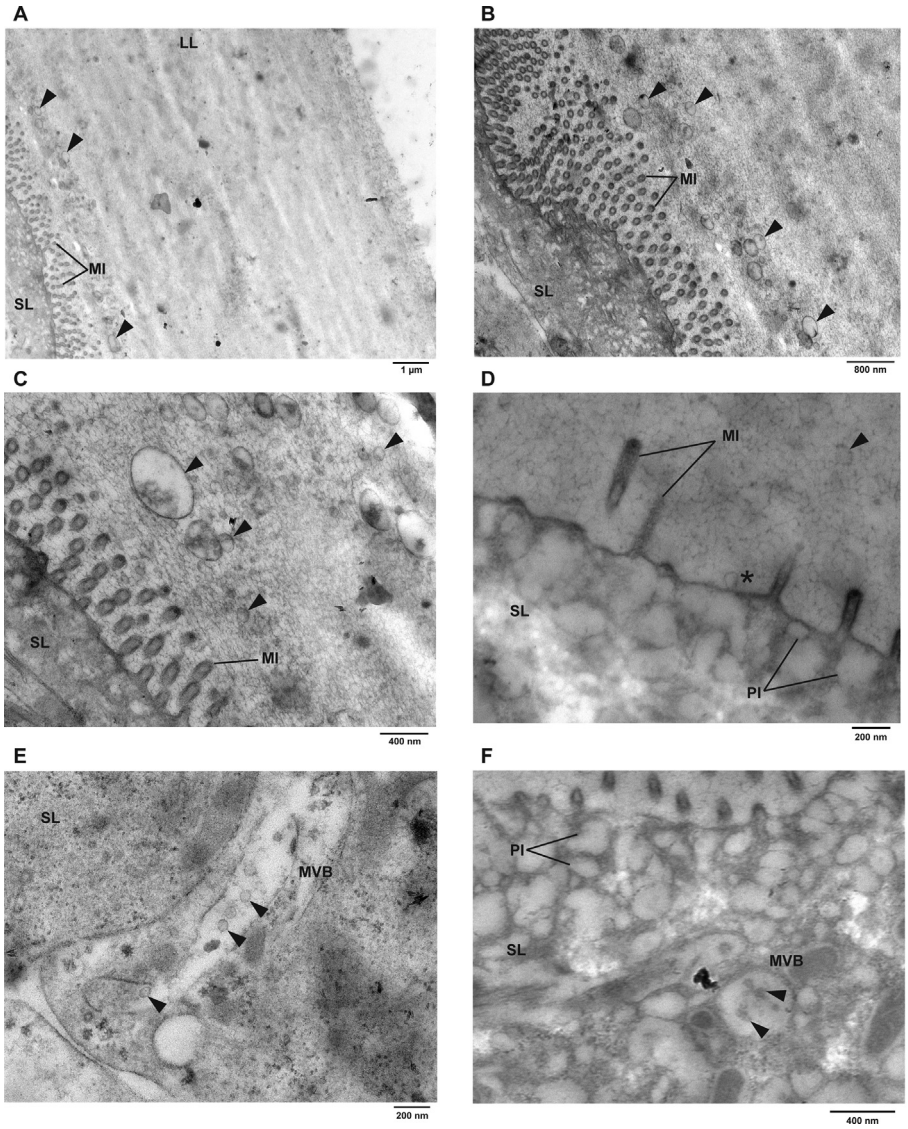

Fig. 3. *Echinococcus multilocularis* secretes extracellular vesicles (EVs). Transmission electron microscopy of the laminated layer (LL) and germinal layer interface (A–D) and tegument (E, F). Arrowheads indicate EVs. The asterisk in D indicates a microvesicle budding from the tegument. MI, microthrix; SL, surface layer.

other platyhelminth vesicles according to our domain search analysis performed on hypothetical or uncharacterised protein sequences (Marcilla et al., 2012; Bernal et al., 2014; Chaiyadet et al., 2015; Cwiklinski et al., 2015; Nowacki et al., 2015; Sotillo et al., 2016) (Supplementary Table S4).

Fatty acid binding proteins (FABPs) and ferlin domain-containing proteins were identified in datasets of both parasites. FABPs have been also identified in EVs from the trematodes *F. hepatica* (Marcilla et al., 2012) and *S. mansoni* (Nowacki et al., 2015). Regarding ferlin domain containing proteins, they act in vesicle trafficking and fusion (Lek et al., 2012) and have been detected in *F. hepatica* vesicles (Cwiklinski et al., 2015).

In addition, antigens highly conserved between *Echinococcus* and *Taenia* and used or tested for echinococcosis and/or cysticercosis immunodiagnosis were detected. Among others, antigen p29 (González et al., 2000) (also annotated under the name endophilin – Supplementary Fig. 2B), FABP (Yang et al., 2013), 14-3-3 (Siles-Lucas et al., 2000), Em18/H17g (here named FERM ezrin/radixin/moesin) (Ito et al., 1993; Deckers and Dorny, 2010) and Ts8B1 (immunodiagnostic antigen) were identified.

Finally, as reported in trematode parasite EVs (Marcilla et al., 2012; Bernal et al., 2014; Cwiklinski et al., 2015), host proteins were detected in the EV-enriched fraction of cestode secreted products. Such proteins corresponded to immunoglobulins and complement factors both in *T. crassiceps* and *M. corti* datasets, as well as albumin and ferritin in *T. crassiceps* (Supplementary Tables S2 and S3).

3.3. Cestode EVs contain small RNAs including miRNAs

The EVs from *T. crassiceps* and *M. corti* contain RNA which is almost exclusively composed of small RNA (<200 nucleotides (nt)) (Fig. 5A). To determine whether the RNA that co-sedimented with the EV fraction in the ultracentrifugation step was actually located intravesicularly, the isolated vesicles were exposed to different treatments. In this way, when the EVs from both parasites were treated with proteinase K followed by RNAse A, the RNA showed the same pattern as the control samples, however, when the samples were exposed also to SDS, the RNA was completely degraded, demonstrating that it was encapsulated, and hence protected, within membranous compartments (Fig. 5A). Additionally, the EV-free fraction from the culture media was concentrated and analysed, and no RNA could be detected (Fig. 5B).

Please cite this article in press as: Ancarola, M.E., et al. Cestode parasites release extracellular vesicles with microRNAs and immunodiagnostic protein cargo. Int. J. Parasitol. (2017), http://dx.doi.org/10.1016/j.ijpara.2017.05.003

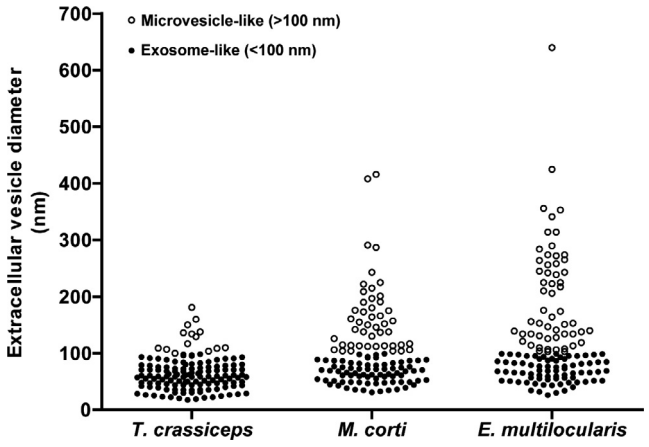

Fig. 4. Cestode extracellular vesicles (EVs) display diameters compatible with those of exosomes and microvesicles. Shown results belong to four biological replicates for *Taenia crassiceps*, three for *Mesocestoides corti* and four for *Echinococcus multilocularis*. Measures correspond to 32, 20 and 21 pictures for *T. crassiceps*, *M. corti* and *E. multilocularis*, respectively. The total numbers of counted EVs were 136, 122 and 131 for *T. crassiceps*, *M. corti* and *E. multilocularis*, respectively.

To investigate the RNA contained in cestode EVs, we searched for the presence of miRNAs. For this, we performed RT-PCR of selected miRNAs which (i) have already been described to be secreted in EVs from trematode and nematode helminth parasites (Bernal et al., 2014; Buck et al., 2014; Fromm et al., 2015), (ii) have been detected in plasma/serum samples of infected hosts (Hoy et al., 2014; Tritten et al., 2014) or (iii) were highly divergent to host miRNAs. The seven selected miRNAs (let-7-5p, miR-61-3p, miR-190-5p, miR-219-5p, miR-4989-3p, miR-71-5p and miR-277-3p) were detected in *T. crassiceps* vesicles, but only let-7-5p was detected within *M. corti* EVs (Fig. 6). Although there was a difference in size between the amplification products from EVs and tetrathyridia, the products in both cases were ~75 bp which corresponded to the expected size.

Finally, an in silico miRNA target search was conducted to predict those mouse transcripts that could be down-regulated by cestode miRNAs upon internalization of the EVs. The candidate targets were functionally annotated with GO terms. Initially, an overall target search was performed taking into consideration the complete repertoire of cestode miRNAs. By doing this, it can be observed that the most putatively regulated pathways in the host

Table 1
Proteins found in extracellular vesicles of both *Taenia crassiceps* and *Mesocestoides corti*.

Proteins present in eukaryotic extracellular vesicles	Other proteins present in cestode extracellular vesicles
Vesicle trafficking	*Antigen/Immunodiagnosis marker*
Annexin	H17g protein, tegumental antigen (FERM ezrin/radixin/moesin)
Myoferlin	p29 (endophilin B1/BAR-domain containing protein)[a]
Otoferlin	Ts8B1[a]
Vacuolar protein sorting associated protein 4A	14-3-3[a]
Rab	
ADP-ribosylation factor	*Vesicle trafficking*
Transforming protein RhoA	Receptor Mediated Endocytosis family member
BRO1 domain containing protein BROX	
Clathrin[a]	*Cytoskeleton*
	Alpha actinin sarcomeric
Cytoskeleton	Actin modulator protein
Actin	
Tubulin	*Signal transduction*
Dynein	Ras gtpase
	Ras protein
Chaperones	Ras-related protein O-RAL
Heat shock 70 kDa	Guanine nucleotide binding protein G(q) subunit
Carrier proteins	*Other*
Ferritin	UPF0047 domain containing protein
Fatty acid binding protein	cGMP dependent protein kinase
	Thioredoxin fold
Metabolism	*Host proteins*
Gyceraldehyde-3-phosphate dehydrogenase	Immunoglobulins
Phosphoenolpyruvate carboxykinase	Complement factors
Cytosolic malate dehydrogenase	
6-phosphogluconate dehydrogenase	
Phosphoglycerate kinase	
Enolase	
RNA binding	
Elongation factor	
Eukaryotic translation initiation factor	
Proteinase	
Calpain	
Synaptic vesicles formation / neurotransmitters exocytosis	
BAR-domain containing protein[a]	
Synaptic vesicle membrane protein VAT 1[a]	
Syntaxin[a]	
Syntaxin-binding protein[a]	
Synaptotagmin[a]	
Synaptobrevin YKT6[b]	
N-ethylmaleimide sensitive factor attachment[a]	
Signal transduction	
Ras protein Rap	
Calcium binding protein	

[a] Only found in the *Taenia crassiceps* dataset.
[b] Only found in the *Mesocestoides corti* dataset.

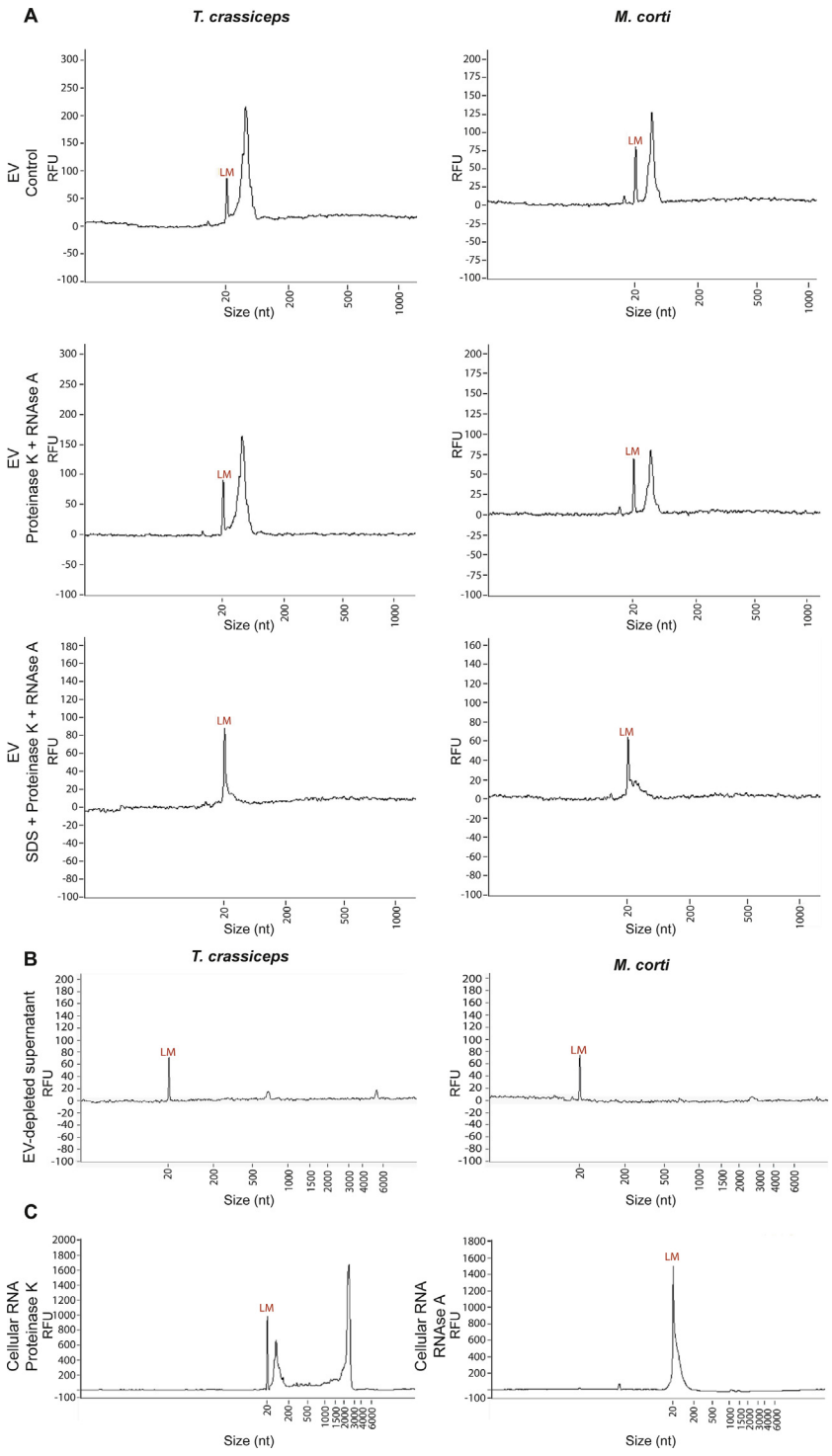

Fig. 5. *Taenia crassiceps* and *Mesocestoides corti* extracellular vesicles (EVs) carry small RNAs (<200 nucleotides (nt)). Capillary electrophoresis analysis of RNA present in EVs (A), EV-free culture supernatants (B) and control cellular RNA (C). RFU, relative fluorescence units; LM, molecular marker. The electropherograms are representative of at least two biological replicates for each parasite.

are those related to Wnt signalling, cadherin signalling, gonadotropin-releasing hormone receptor, inflammation mediated by chemokine and cytokine signalling and angiogenesis (Fig. 7A). With respect to the subset of transcripts that (i) may be regulated by the secreted miRNAs which share 100% sequence identity among *Echinococcus* spp., *Taenia* spp. and *M. corti* (Supplementary Fig. S3), and (ii) display more than one miRNA binding site, thus enhancing the stringency of the prediction, it could be observed that the main regulated pathways again involve Wnt and cadherin signalling but also transcripts sorted in six other pathways mainly related to the immune response (Fig. 7B; Supplementary Table S5).

4. Discussion

In this work we describe for the first time, the in vitro secretion of EVs in cestode parasites based on (i) the detection of secreted

Please cite this article in press as: Ancarola, M.E., et al. Cestode parasites release extracellular vesicles with microRNAs and immunodiagnostic protein cargo. Int. J. Parasitol. (2017), http://dx.doi.org/10.1016/j.ijpara.2017.05.003

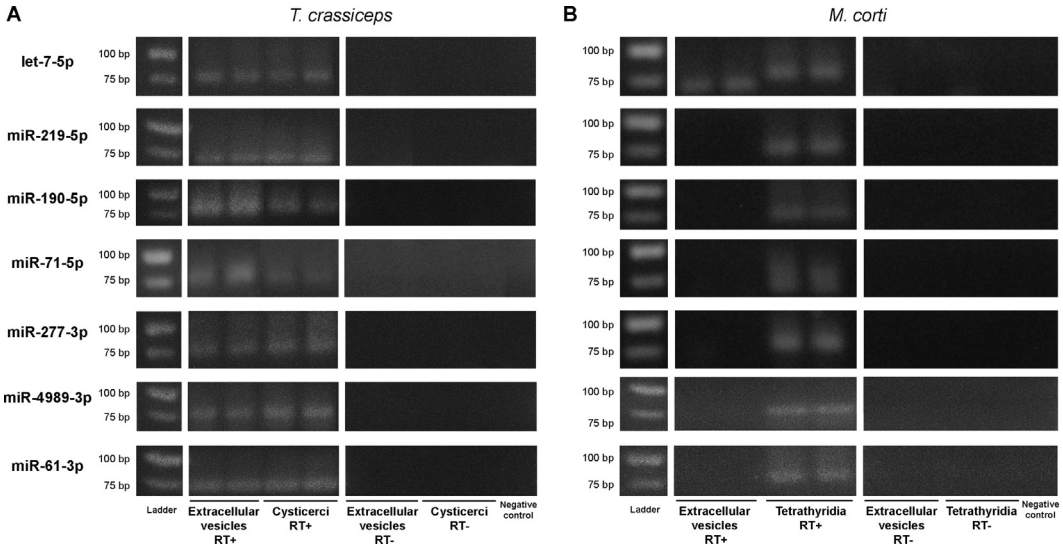

Fig. 6. *Taenia crassiceps* and *Mesocestoides corti* extracellular vesicles (EVs) carry microRNAs (miRNAs). Reverse transcription (RT)-PCR detection of miRNA expression in EVs and cysticerci (positive control) of *T. crassiceps* (A). RT-PCR detection of *M. corti* miRNAs in EVs and tetrathyridia samples (positive control) (B). RT+, cDNA samples; RT−, no reverse transcriptase control. Negative control, PCR mix.

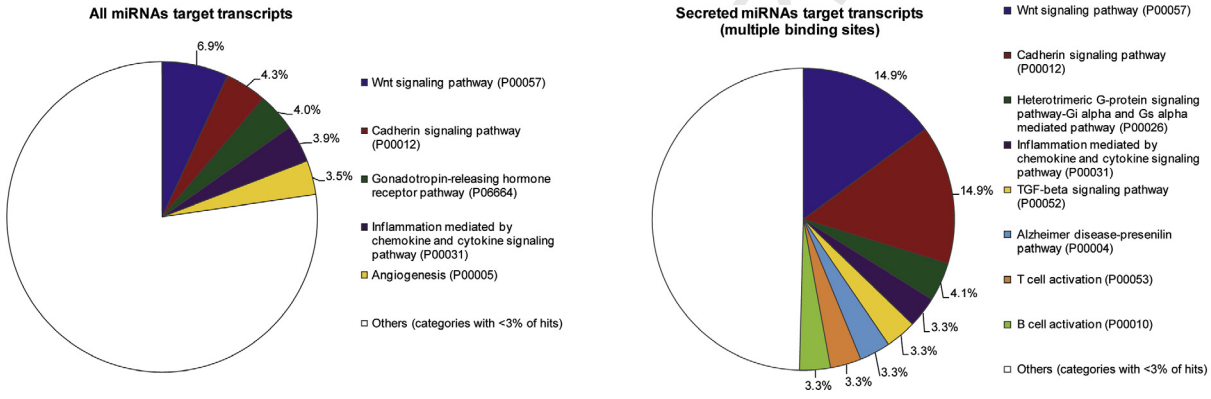

Fig. 7. Functional annotation of mouse transcripts putatively targeted by cestode microRNAs (miRNAs). Annotation was performed using Pantherdb Pathway classification.

membrane-bound structures, of which the morphology and size are in accordance with those reported for exosomes and microvesicles; (ii) the presence of multivesicular body-like complexes in parasite tissue; (iii) the identification of proteins reported to be present in EVs from model organisms (mouse and human); (iv) the detection of a specific class of RNA (small RNAs) carried within these subcellular particles and (iv) the conserved ultrastructural detection of these vesicles in three species of cestodes which belong to two different families (Taeniidae and Mesocestoididae). Furthermore, this work constitutes the first report on a nucleic acid secretion mechanism in this class of platyhelminths.

It is highly remarkable that even though the metacestode stages of the three species produce and secrete EVs, only those secreted by *T. crassiceps* and *M. corti* would be in direct contact with the host since the laminated layer of *E. multilocularis*, when intact, seems to act as a barrier for such large structures, at least under the studied conditions. The laminated layer is a specialized extracellular matrix found only in the genus *Echinococcus*. It confers physical integrity to the metacestodes and protects the germinal layer cells from the host immune response (Díaz et al., 2011). Our findings are in agreement with previous ultrastructural observations in *Echinococcus* spp. (Lascano et al., 1975; Ingold et al., 2000, 2001), where membrane-bound structures can be observed only in the proximity of the germinal layer. This result suggests that the EVs may be in contact with the host in the early stages of development, when the laminated layer is still not formed or incipient, and/or when the laminated layer undergoes rupture due to metacestode ageing or chemotherapy treatment. In line with the first, in an ultrastructural study of the development of the tegument of *E. granulosus* sensu lato in the protoscolex-metacestode transition (Rogan and Richards, 1989), the presence of EV-like structures in the outer layers of the laminated layer of early forming cysts could be observed. On the other hand, these vesicles may contain the components needed for the laminated layer formation, in addition to the exocytic vesicles already described by Rogan and Richards (1989). Our results suggest that even though the secretion of EVs seems to be a conserved mechanism in cestode parasites from different genera or families, the particular traits of each parasite may confer specific roles to these subcellular particles.

It is worth mentioning that not only conventional EVs may have been isolated using the differential centrifugation approach. The possibility that other components are present in our preparations cannot be totally discarded. However, there are reports on other non-conventional membrane secreted particles such as in sperm (Höög and Lotvall, 2015), which reflects the fact that we are beginning to understand the great diversity found among EVs.

With respect to the proteins present in the EV-enriched secreted fractions of cestode-conditioned media, most of the

Please cite this article in press as: Ancarola, M.E., et al. Cestode parasites release extracellular vesicles with microRNAs and immunodiagnostic protein cargo. Int. J. Parasitol. (2017), http://dx.doi.org/10.1016/j.ijpara.2017.05.003

identified proteins have been reported in mouse and/or human EVs, regardless of whether those were exosomes or microvesicles. Since a gradient separation technique was not used for the EV purification, we might therefore be dealing with multiple populations, which is reflected in both the TEM and proteomics results. Furthermore, we cannot disregard the presence of contaminating soluble proteins secreted by the parasites.

In particular, clathrin was detected in *T. crassiceps* EVs, which is in agreement with a previous ultrastructural report that describes the presence of clathrin-coated pit-like structures in *T. crassiceps* cysticerci (Threadgold and Dunn, 1983) that are formed during clathrin-dependent endocytosis (Gould and Lippincott-Schwartz, 2009). Since exosomes are formed by inward budding of the early endosomal membrane, thus yielding multivesicular bodies (Colombo et al., 2014), the presence of this protein gives further support to the obtained results.

Remarkably, antigens tested for echinococcosis and/or cysticercosis immunodiagnosis were identified in cestode EVs. According to the obtained results, the identification of antigenic proteins in EVs may aid in developing a more rational approach for selecting diagnostic candidates according to the infecting species. In the case of confirming that *Echinococcus* spp. secrete these antigens in EVs, which is likely since both *T. crassiceps* and *M. corti* do, these antigens would be released in the initial stages of establishment of the parasite or in the case of fissure of the laminated layer. Here we also provide experimental evidence for the actual expression and secretion of proteins previously annotated as "hypothetical", which may represent new diagnostic targets.

Previous reports showed the presence of FABPs in cysticercal excretion/secretion products (Victor et al., 2012), hydatid fluid (Aziz et al., 2011) and excretion/secretion products of protoscoleces of *E. granulosus* sensu lato (Virginio et al., 2012). In this work we determined that *T. crassiceps* and *M. corti* secrete FABPs in EVs. Members of the FABPs family have been identified in exosomes from mouse adipocytes and macrophages in vitro (Hotamisligil and Bernlohr, 2015). Interestingly, secreted FABPs have been reported to enhance hepatic glucose production (Hotamisligil and Bernlohr, 2015). Since cestode parasites are unable to synthesize lipids de novo (Tsai et al., 2013), it would be interesting to analyse whether the secretion of FABPs in EVs is a means of scavenging host fatty acids and cholesterol or if their role is to induce glucose production.

Host proteins were also detected in both parasites samples. In this early study we cannot confidently confirm that these proteins are actively packaged into cestode EVs. However, it would be highly interesting to determine whether the presence of immunoglobulins in the cestode EV-enriched fractions represent a mechanism of the parasites to remove, by endocytosis, host damaging molecules attached to the tegument according to the nutritional-protective role proposed for cestode tegument (Bereiter-Hahn et al., 1984). Another host protein present in the *T. crassiceps* dataset was albumin. It has been reported that albumin is a strong inductor of endocytosis in the metacestode stage of this parasite (Threadgold and Dunn, 1984), thus it is likely there will be albumin in the exosomes secreted by cysticerci. In addition, it has been reported that host albumin is not only actively internalized but also secreted by *T. crassiceps* (Aldridge et al., 2006). However, as mentioned before, further assays are needed to confirm its presence within *T. crassiceps* EVs.

With respect to the nucleic acid content of the EVs, we demonstrated the presence of vesicular RNA. In this case, as in those reported for protozoan and helminth parasites (Twu et al., 2013; Buck et al., 2014; Lambertz et al., 2015), the detected RNA corresponded to small RNAs <200 nt according to their size distribution pattern, while in mammalian EVs RNA >200 nt has been also detected (Valadi et al., 2007; Crescitelli et al., 2013). However, more sensitive assays should be performed to conclusively discard the intravesicular presence of mRNA. Among the small RNA species present intravesicularly, we detected miRNAs. Although we cannot establish whether miRNAs represent the main secreted RNA population since a global analysis by high-throughput small RNA-sequencing should be performed to reach this conclusion, we can confirm the extracellular nature of the detected miRNAs. In this respect, we observed that the profile of the identified miRNAs varied between *T. crassiceps* and *M. corti*, which suggests a differential secretion pattern between species. Since most of the miRNAs detected were chosen according to current data on miRNA secretion in nematodes and trematodes, it seems that there is at least a set of these small RNAs which are commonly secreted by helminth parasites. This may reflect a common role in, for instance, host immune response regulation since the internalization of helminth parasitic vesicles by host cells was shown in vitro (Marcilla et al., 2012; Buck et al., 2014; Chaiyadet et al., 2015; Zamanian et al., 2015), as well as the down-regulation of host genes involved in the pro-inflammatory response (Buck et al., 2014). In this respect, a miRNA target prediction on mice transcripts yielded that transcripts related to signalling processes and inflammation would be likely regulated by cestode-secreted miRNAs.

In conclusion, here we report the existence of an intercellular communication mechanism in cestode parasites which provides valuable data not only for basic cestode biology but also for the rational search for new diagnostic targets.

Acknowledgements

This work was supported by Fundación Bunge y Born, Grant for Research on Infectious Diseases and Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), Argentina, projects PICT 2013N° 2121 and 2014N°1138 and Conselleria d'Educació, Cultura i Esports, Generalitat Valenciana (Valencia, Spain) (Ref. PROMETEO/2016/156). The authors would like to thank Dr. Andrea Peralta for assisting with the use of the ultracentrifuge at Instituto de Biotecnología, Centro de Investigación en Ciencias Veterinarias y Agronómicas (CICVyA) – Instituto Nacional de Tecnología Agropecuaria (INTA), Argentina.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpara.2017.05.003.

References

Aldridge, J.R., Jennette, M.a., Kuhn, R.E., 2006. Uptake and secretion of host proteins by *Taenia crassiceps* metacestodes. J. Parasitol. 92, 1101–1102. http://dx.doi.org/10.1645/GE-835R.1.

Arroyo, J.D., Chevillet, J.R., Kroh, E.M., Ruf, I.K., Pritchard, C.C., Gibson, D.F., Mitchell, P.S., Bennett, C.F., Pogosova-Agadjanyan, E.L., Stirewalt, D.L., Tait, J.F., Tewari, M., 2011. Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. Proc. Natl. Acad. Sci. U.S.A. 108, 5003–5008. http://dx.doi.org/10.1073/pnas.1019055108.

Aziz, A., Zhang, W., Li, J., Loukas, A., McManus, D.P., Mulvenna, J., 2011. Proteomic characterisation of *Echinococcus granulosus* hydatid cyst fluid from sheep, cattle and humans. J. Proteomics 74, 1560–1572. http://dx.doi.org/10.1016/j.jprot.2011.02.021.

Basika, T., Macchiaroli, N., Cucher, M., Espínola, S., Kamenetzky, L., Zaha, A., Rosenzvit, M., Ferreira, H., 2016. Identification and profiling of microRNAs in two developmental stages of the model cestode parasite *Mesocestoides corti*. Mol. Biochem. Parasitol. 210, 37–49.

Bereiter-Hahn, J., Matoltsy, A., Richards, K. (Eds.), 1984. Biology of the Integument – 1 Invertebrates. Springer Berlin Heidelberg, Berlin Heidelberg New York Tokyo. http://dx.doi.org/10.1007/978-3-642-51593-4.

Bernal, D., Trelis, M., Montaner, S., Cantalapiedra, F., Galiano, A., Hackenberg, M., Marcilla, A., 2014. Surface analysis of *Dicrocoelium dendriticum*. The molecular

characterization of exosomes reveals the presence of miRNAs. J. Proteomics 105, 232–241. http://dx.doi.org/10.1016/j.jprot.2014.02.012.
Buck, A.H., Coakley, G., Simbari, F., McSorley, H.J., Quintana, J.F., Le Bihan, T., Kumar, S., Abreu-Goodger, C., Lear, M., Harcus, Y., Ceroni, A., Babayan, S.a., Blaxter, M., Ivens, A., Maizels, R.M., 2014. Exosomes secreted by nematode parasites transfer small RNAs to mammalian cells and modulate innate immunity. Nat. Commun. 5, 5488. http://dx.doi.org/10.1038/ncomms6488.
Chaiyadet, S., Sotillo, J., Smout, M., Cantacessi, C., Jones, M.K., Johnson, M.S., Turnbull, L., Whitchurch, C.B., Potriquet, J., Laohaviroj, M., Mulvenna, J., Brindley, P.J., Bethony, J.M., Laha, T., Sripa, B., Loukas, A., 2015. Carcinogenic liver fluke secretes extracellular vesicles that promote cholangiocytes to adopt a tumorigenic phenotype. J. Infect. Dis. 212, 1636–1645. http://dx.doi.org/10.1093/infdis/jiv291.
Colombo, M., Raposo, G., Théry, C., 2014. Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. Annu. Rev. Cell Dev. Biol 30, 255–289. http://dx.doi.org/10.1146/annurev-cellbio-101512-122326.
Crescitelli, R., Lässer, C., Szabo, T.G., Kittel, A., Eldh, M., Dianzani, I., Buzás, E.I., Lötvall, J., 2013. Distinct RNA profiles in subpopulations of extracellular vesicles: apoptotic bodies, microvesicles and exosomes. J. Extracell. Vesicles 2, 1–10. http://dx.doi.org/10.3402/jev.v2i0.20677.
Cucher, M., Macchiaroli, N., Kamenetzky, L., Maldonado, L., Brehm, K., Rosenzvit, M., 2015. High-throughput characterization of *Echinococcus* spp. metacestode miRNomes. Int. J. Parasitol. 45, 253–267. http://dx.doi.org/10.1016/j.ijpara.2014.12.003.
Cwiklinski, K., de la Torre-Escudero, E., Trelis, M., Bernal, D., Dufresne, P.J., Brennan, G.P., O'Neill, S., Tort, J., Paterson, S., Marcilla, A., Dalton, J.P., Robinson, M.W., 2015. The extracellular vesicles of the helminth pathogen, fasciola hepatica : biogenesis pathways and cargo molecules involved in parasite pathogenesis. Mol. Cell. Proteomics 14, 3258–3273. http://dx.doi.org/10.1074/mcp.M115.053934.
Deckers, N., Dorny, P., 2010. Immunodiagnosis of *Taenia solium* taeniosis/cysticercosis. Trends Parasitol. 26, 137–144. http://dx.doi.org/10.1016/j.pt.2009.12.008.
Díaz, A., Casaravilla, C., Irigoín, F., Lin, G., Previato, J., Ferreira, F., 2011. Understanding the laminated layer of larval *Echinococcus* I: structure. Trends Parasitol. 27, 204–213.
Enright, A.J., John, B., Gaul, U., Tuschl, T., Sander, C., Marks, D.S., 2003. MicroRNA targets in *Drosophila*. Genome Biol. 5, 1–14.
Fromm, B., Trelis, M., Hackenberg, M., Cantalapiedra, F., Bernal, D., Marcilla, A., 2015. The revised microRNA complement of *Fasciola hepatica* reveals a plethora of overlooked microRNAs and evidence for enrichment of immuno-regulatory microRNAs in extracellular vesicles. Int. J. Parasitol 45, 697–702. http://dx.doi.org/10.1016/j.ijpara.2015.06.002.
Galán-Puchades, M.T., Yang, Y., Marcilla, A., Choe, S., Park, H., Osuna, A., Eom, K.S., 2016. First ultrastructural data on the human tapeworm Taenia asiatica eggs by scanning and transmission electron microscopy (SEM, TEM). Parasitol. Res. http://dx.doi.org/10.1007/s00436-016-5165-4.
Gardiner, C., Vizio, D..Di., Sahoo, S., The, C., Witwer, K.W., Wauben, M., Hill, A.F., 2016. Techniques used for the isolation and characterization of extracellular vesicles: results of a worldwide survey. J. Extracell. Vesicles 5, 32945–32950. http://dx.doi.org/10.3402/jev.v5.32945.
Geer, L.Y., Domrachev, M., Lipman, D.J., Bryant, S.H., 2002. CDART: Protein homology by domain architecture. Genome Res. 12, 1619–1623. http://dx.doi.org/10.1101/gr.278202.
Geiger, A., Hirtz, C., Bécue, T., Bellard, E., Centeno, D., Gargani, D., Rossignol, M., Cuny, G., Peltier, J.-B., 2010. Exocytosis and protein secretion in *Trypanosoma*. BMC Microbiol. 10, 20. http://dx.doi.org/10.1186/1471-2180-10-20.
González, G., Spinelli, P., Lorenzo, C., Hellman, U., Nieto, A., Willis, A., Salinas, G., 2000. Molecular characterization of P-29, a metacestode-specific component of *Echinococcus granulosus* which is immunologically related to, but distinct from, antigen 5. Mol. Biochem. Parasitol. 105, 177–184. http://dx.doi.org/10.1016/S0166-6851(99)00166-8.
Gould, G.W., Lippincott-Schwartz, J., 2009. New roles for endosomes: from vesicular carriers to multi-Purpose Platforms. Nat. Rev. Mol. Cell Biol. 10, 287–292. http://dx.doi.org/10.1038/nrm2652.New.
Hansen, E.P., Kringel, H., Williams, A.R., Nejsum, P., 2015. Secretion of RNA-containing extracellular vesicles by the porcine whipworm, *Trichuris suis*. J. Parasitol. 101, 336–340. http://dx.doi.org/10.1645/14-714.1.
Höög, J.L., Lotvall, J., 2015. Diversity of extracellular vesicles in human ejaculates revealed by cryo-electron microscopy. J. Extracell. Vesicles 4, 28680. http://dx.doi.org/10.3402/jev.v4.28680.
Hotamisligil, G.S., Bernlohr, D.A., 2015. Metabolic functions of FABPs-mechanisms and therapeutic implications. Nat. Rev. Endocrinol. 11, 592–605. http://dx.doi.org/10.1038/nrendo.2015.122.
Hoy, A.M., Lundie, R.J., Ivens, A., Quintana, J.F., Nausch, N., Forster, T., Jones, F., Kabatereine, N.B., Dunne, D.W., Mutapi, F., Macdonald, A.S., Buck, A.H., 2014. Parasite-derived microRNAs in host serum as novel biomarkers of helminth infection. PLoS Negl. Trop. Dis. 8, e2701. http://dx.doi.org/10.1371/journal.pntd.0002701.
Ingold, K., Dai, W., Rausch, R.L., Gottstein, B., Hemphill, A., 2001. Characterization of the laminated layer of in vitro cultivated *Echinococcus vogeli* metacestodes. J. Parasitol. 87, 55–64. http://dx.doi.org/10.1645/0022-3395(2001) 087[0055:COTLLO]2.0.CO;2.
Ingold, K., Gottstein, B., Hemphill, A., 2000. High molecular mass glycans are major structural elements associated with the laminated layer of in vitro cultivated *Echinococcus multilocularis* metacestodes. Int. J. Parasitol. 30, 207–214. http://dx.doi.org/10.1016/S0020-7519(99)00177-0.
Ito, A., Nakao, M., Kutsumi, H., Lightowlers, M.W., Itoh, M., Sato, S., 1993. Serodiagnosis of alveolar hydatid disease by Western blotting. Trans. R. Soc. Trop. Med. Hyg. 87, 170–172. http://dx.doi.org/10.1016/0035-9203(93)90476-7.
Jones, D.T., Taylor, W.R., Thornton, J.M., 1992. The rapid generation of mutation data matrices from protein sequences. Bioinformatics 8, 275–282. http://dx.doi.org/10.1093/bioinformatics/8.3.275.
Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Mol. Biol. Evol., msw054 http://dx.doi.org/10.1093/molbev/msw054.
Lambertz, U., Oviedo Ovando, M.E., Vasconcelos, E.J.R., Unrau, P.J., Myler, P.J., Reiner, N.E., 2015. Small RNAs derived from tRNAs and rRNAs are highly enriched in exosomes from both old and new world *Leishmania* providing evidence for conserved exosomal RNA Packaging. BMC Genomics 16, 151. http://dx.doi.org/10.1186/s12864-015-1260-7.
Lascano, E.F., Coltorti, E.A., Varela-Díaz, V.M., 1975. Fine structure of the germinal membrane of *Echinococcus granulosus* cysts. J. Parasitol. 61, 853–860.
Lek, A., Evesson, F.J., Sutton, R.B., North, K.N., Cooper, S.T., 2012. Ferlins: regulators of vesicle fusion for auditory neurotransmission, receptor trafficking and membrane repair. Traffic 13, 185–194. http://dx.doi.org/10.1111/j.1600-0854.2011.01267.x.
Lötvall, J., Hill, A.F.A.F., Hochberg, F., Buzás, E.I.E.I., Di Vizio, D., Gardiner, C., Gho, Y.S. Y.S., Kurochkin, I.V.I.V., Mathivanan, S., Quesenberry, P., Sahoo, S., Tahara, H., Wauben, M.H.M.H., Witwer, K.W.K.W., Théry, C., 2014. Minimal experimental requirements for definition of extracellular vesicles and their functions: a position statement from the International Society for Extracellular Vesicles. J. Extracell. Vesicles 3, 26913. http://dx.doi.org/10.3402/jev.v3.26913.
Macchiaroli, N., Cucher, M., Zarowiecki, M., Maldonado, L., Kamenetzky, L., Rosenzvit, M.C., 2015. MicroRNA profiling in the zoonotic parasite *Echinococcus canadensis* using a high-throughput approach. Parasit. Vectors 8, 83. http://dx.doi.org/10.1186/s13071-015-0686-8.
Maldonado, L.L., Assis, J., Araújo, F.M.G., Salim, A.C.M., Macchiaroli, N., Cucher, M., Camicia, F., Fox, A., Rosenzvit, M., Oliveira, G., Kamenetzky, L., 2017. The *Echinococcus canadensis* (G7) genome: a key knowledge of parasitic platyhelminth human diseases. BMC Genomics 18, 204. http://dx.doi.org/10.1186/s12864-017-3574-0.
Marcilla, A., Trelis, M., Cortés, A., Sotillo, J., Cantalapiedra, F., Minguez, M.T., Valero, M.L., Sánchez del Pino, M.M., Muñoz-Antoli, C., Toledo, R., Bernal, D., 2012. Extracellular vesicles from parasitic helminths contain specific excretory/secretory proteins and are internalized in intestinal host cells. PLoS One 7, e45974. http://dx.doi.org/10.1371/journal.pone.0045974.
Mi, H., Poudel, S., Muruganujan, A., Casagrande, J.T., Thomas, P.D., 2016. PANTHER version 10: expanded protein families and functions, and analysis tools. Nucleic Acids Res. 44, D336–42. http://dx.doi.org/10.1093/nar/gkv1194.
Montecalvo, A., Larregina, A.T., Shufesky, W.J., Stolz, D.B., Sullivan, M.L.G., Karlsson, J.M., Baty, C.J., Gibson, G.A., Erdos, G., Wang, Z., Milosevic, J., Tkacheva, O.A., Divito, S.J., Jordan, R., Lyons-Weiler, J., Watkins, S.C., Morelli, A.E., 2012. Mechanism of transfer of functional microRNAs between mouse dendritic cells via exosomes. Blood 119, 756–766. http://dx.doi.org/10.1182/blood-2011-02-338004.
Munson, M., 2015. Synaptic-vesicle fusion: a need for speed. Nat. Struct. Mol. Biol. 22, 509–511. http://dx.doi.org/10.1038/nsmb.3056.
Nowacki, F.C., Swain, M.T., Klychnikov, O.I., Niazi, U., Ivens, A., Quintana, J.F., Hensbergen, P.J., Hokke, C.H., Buck, A.H., Hoffmann, K.F., 2015. Protein and small non-coding RNA-enriched extracellular vesicles are released by the pathogenic blood fluke *Schistosoma mansoni*. J. Extracell. Vesicles 4, 1–16. http://dx.doi.org/10.3402/jev.v4.28665.
Petersen, T.N., Brunak, S., von Heijne, G., Nielsen, H., 2011. SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat. Methods 8, 785–786. http://dx.doi.org/10.1038/nmeth.1701.
Regev-Rudzki, N., Wilson, D.W., Carvalho, T.G., Sisquella, X., Coleman, B.M., Rug, M., Bursac, D., Angrisano, F., Gee, M., Hill, A.F., Baum, J., Cowman, A.F., 2013. Cell-cell communication between malaria-infected red blood cells via exosome-like vesicles. Cell 153, 1120–1133. http://dx.doi.org/10.1016/j.cell.2013.04.029.
Rogan, M.T., Richards, K.S., 1989. Development of the tegument of *Echinococcus granulosus* (Cestoda) protoscoleces during cystic differentiation in vivo. Parasitol. Res. 75, 299–306. http://dx.doi.org/10.1007/BF00931814.
Shelke, G.V., Lässer, C., Gho, Y.S., Lötvall, J., 2014. Importance of exosome depletion protocols to eliminate functional and RNA-containing extracellular vesicles from fetal bovine serum. J. Extracell. Vesicles 3, 24783. http://dx.doi.org/10.3402/jev.v3.24783.
Shilov, I.V., Seymour, S.L., Patel, A.A., Loboda, A., Tang, W.H., Keating, S.P., Hunter, C. L., Nuwaysir, L.M., Schaeffer, D., a, 2007. The Paragon Algorithm, a next generation search engine that uses sequence temperature values and feature probabilities to identify peptides from tandem mass spectra. Mol. Cell. Proteomics 6, 1638–1655. http://dx.doi.org/10.1074/mcp.T600050-MCP200.
Siles-Lucas, M., Nunes, C.P., Zaha, A., Breijo, M., 2000. The 14–3-3 protein is secreted by the adult worm of Echinococcus granulosus. Parasite Immunol. 22, 521–528. http://dx.doi.org/10.1046/j.1365-3024.2000.00334.x.
Silverman, J.M., Clos, J., Horakova, E., Wang, A.Y., Wiesgigl, M., Kelly, I., Lynn, M.A., McMaster, W.R., Foster, L.J., Levings, M.K., Reiner, N.E., 2010. Leishmania exosomes modulate innate and adaptive immune responses through effects on monocytes and dendritic cells. J Immunol 185, 5011–5022. http://dx.doi.org/10.4049/jimmunol.1000541.

Please cite this article in press as: Ancarola, M.E., et al. Cestode parasites release extracellular vesicles with microRNAs and immunodiagnostic protein cargo. Int. J. Parasitol. (2017), http://dx.doi.org/10.1016/j.ijpara.2017.05.003

Sotillo, J., Pearson, M., Potriquet, J., Becker, L., Pickering, D., Mulvenna, J., Loukas, A., 2016. Extracellular vesicles secreted by *Schistosoma mansoni* contain protein vaccine candidates. Int. J. Parasitol. 46, 1–5. http://dx.doi.org/10.1016/j.ijpara.2015.09.002.

Spiliotis, M., Brehm, K., 2009. Axenic in vitro cultivation of *Echinococcus multilocularis* metacestode vesicles and the generation of primary cell cultures. Methods Mol. Biol. 470, 245–262. http://dx.doi.org/10.1007/978-1-59745-204-5{_}17.

Théry, C., Amigorena, S., Raposo, G., Clayton, A., 2006. Isolation and characterization of exosomes from cell culture supernatants. Curr. Protoc. Cell Biol. Chapter 3, Unit 3.22. http://dx.doi.org/10.1002/0471143030.cb0322s30.

Threadgold, L., Dunn, J., 1984. *Taenia crassiceps*: basic mechanisms of endocytosis in the cysticercus. Exp. Parasitol. 58, 263–269.

Threadgold, L.T., Dunn, J., 1983. Taenia crassiceps : Regional Variations in Ultrastructure and evidence of endocytosis in the Cysticercus ' Tegument. Exp. Parasitol. 55, 121–131.

Tritten, L., Burkman, E., Moorhead, A., Satti, M., Geary, J., Mackenzie, C., Geary, T., 2014. Detection of circulating parasite-derived microRNAs in filarial infections. PLoS Negl. Trop. Dis. 8, e2971. http://dx.doi.org/10.1371/journal.pntd.0002971.

Tsai, I.J., Zarowiecki, M., Holroyd, N., Garciarrubio, A., Sanchez-Flores, A., Brooks, K. L., Tracey, A., Bobes, R.J., Fragoso, G., Sciutto, E., Aslett, M., Beasley, H., Bennett, H.M., Cai, J., Camicia, F., Clark, R., Cucher, M., De Silva, N., Day, T.A., Deplazes, P., Estrada, K., Fernández, C., Holland, P.W.H., Hou, J., Hu, S., Huckvale, T., Hung, S.S., Kamenetzky, L., Keane, J.A., Kiss, F., Koziol, U., Lambert, O., Liu, K., Luo, X., Luo, Y., Macchiaroli, N., Nichol, S., Paps, J., Parkinson, J., Pouchkina-Stantcheva, N., Riddiford, N., Rosenzvit, M., Salinas, G., Wasmuth, J.D., Zamanian, M., Zheng, Y., Cai, X., Soberón, X., Olson, P.D., Laclette, J.P., Brehm, K., Berriman, M., 2013. The genomes of four tapeworm species reveal adaptations to parasitism. Nature 496, 57–63. http://dx.doi.org/10.1038/nature12031.

Twu, O., de Miguel, N., Lustig, G., Stevens, G.C., Vashisht, A.A., Wohlschlegel, J.A., Johnson, P.J., 2013. Trichomonas vaginalis exosomes deliver cargo to host cells and mediate host: parasite interactions. PLoS Pathog. 9, 22–24. http://dx.doi.org/10.1371/journal.ppat.1003482.

Tzelos, T., Matthews, J.B., Buck, A.H., Simbari, F., Frew, D., Inglis, N.F., McLean, K., Nisbet, A.J., Whitelaw, C.B.A., Knox, D.P., McNeilly, T.N., 2016. A preliminary proteomic characterisation of extracellular vesicles released by the ovine parasitic nematode, *Teladorsagia circumcincta*. Vet. Parasitol. 221, 84–92. http://dx.doi.org/10.1016/j.vetpar.2016.03.008.

Valadi, H., Ekström, K., Bossios, A., Sjöstrand, M., Lee, J.J., Lötvall, J.O., 2007. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat. Cell Biol. 9, 654–659. http://dx.doi.org/10.1038/ncb1596.

Vickers, K.C., Palmisano, B.T., Shoucri, B.M., Shamburek, R.D., Remaley, A.T., 2011. MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins. Nat. Cell Biol. 13, 423–433. http://dx.doi.org/10.1038/ncb2210.

Victor, B., Kanobana, K., Gabriël, S., Polman, K., Deckers, N., Dorny, P., Deelder, A.M., Palmblad, M., 2012. Proteomic analysis of *Taenia solium* metacestode excretion-secretion proteins. Proteomics 12, 1860–1869. http://dx.doi.org/10.1002/pmic.201100496.

Virginio, V.G., Monteiro, K.M., Drumond, F., De Carvalho, M.O., Vargas, D.M., Zaha, A., Ferreira, H.B., 2012. Excretory/secretory products from in vitro-cultured *Echinococcus granulosus* protoscoleces. Mol. Biochem. Parasitol. 183, 15–22. http://dx.doi.org/10.1016/j.molbiopara.2012.01.001.

Wang, L., Li, Z., Shen, J., Liu, Z., Liang, J., Wu, X., Sun, X., Wu, Z., 2015. Exosome-like vesicles derived by *Schistosoma japonicum* adult worms mediates M1 type immune- activity of macrophage. Parasitol. Res., 1865–1873 http://dx.doi.org/10.1007/s00436-015-4373-7.

Wu, X., Fu, Y., Yang, D., Xie, Y., Zhang, R., Zheng, W., Nie, H., Yan, N., Wang, N., Wang, J., Gu, X., Wang, S., Peng, X., Yang, G., 2013. Identification of neglected cestode *Taenia multiceps* microRNAs by illumina sequencing and bioinformatic analysis. BMC Vet. Res. 9, 162. http://dx.doi.org/10.1186/1746-6148-9-162.

Yang, D., Chen, L., Xie, Y., Wu, X., Nong, X., Peng, X., Lai, W., Gu, X., Wang, S., Peng, X., Yang, G., 2013. Expression and immunolocalisation of TpFABP as a candidate antigen for the serodiagnosis of rabbit *Taenia pisiformis* cysticercosis. Parasite 20, 53. http://dx.doi.org/10.1051/parasite/2013053.

Yates, A., Akanni, W., Amode, M.R., Barrell, D., Billis, K., Carvalho-Silva, D., Cummins, C., Clapham, P., Fitzgerald, S., Gil, L., Girón, C.G., Gordon, L., Hourlier, T., Hunt, S. E., Janacek, S.H., Johnson, N., Juettemann, T., Keenan, S., Lavidas, I., Martin, F.J., Maurel, T., McLaren, W., Murphy, D.N., Nag, R., Nuhn, M., Parker, A., Patricio, M., Pignatelli, M., Rahtz, M., Riat, H.S., Sheppard, D., Taylor, K., Thormann, A., Vullo, A., Wilder, S.P., Zadissa, A., Birney, E., Harrow, J., Muffato, M., Perry, E., Ruffier, M., Spudich, G., Trevanion, S.J., Cunningham, F., Aken, B.L., Zerbino, D.R., Flicek, P., 2016. Ensembl 2016. Nucleic Acids Res. 44, D710–D716. http://dx.doi.org/10.1093/nar/gkv1157.

Zamanian, M., Fraser, L.M., Agbedanu, P.N., Harischandra, H., Moorhead, A.R., Day, T. A., Bartholomay, L.C., Kimber, M.J., 2015. Release of small RNA-containing exosome-like vesicles from the human filarial parasite *Brugia malayi*. PLoS Negl. Trop. Dis. 9, 1–23. http://dx.doi.org/10.1371/journal.pntd.0004069.

Please cite this article in press as: Ancarola, M.E., et al. Cestode parasites release extracellular vesicles with microRNAs and immunodiagnostic protein cargo. Int. J. Parasitol. (2017), http://dx.doi.org/10.1016/j.ijpara.2017.05.003