International Journal for Parasitology 50 (2020) 635–645

Contents lists available at ScienceDirect

International Journal for Parasitology

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

Invited Review

The protein and microRNA cargo of extracellular vesicles from parasitic helminths – current status and research priorities

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ARTICLE INFO

Article history:
Received 3 February 2020
Received in revised form 18 April 2020
Accepted 21 April 2020
Available online 8 July 2020

Keywords:
Helminths
Parasites
Extracellular vesicles
Exosomes
Microvesicles
Protein
MicroRNA
Cargo

ABSTRACT

Helminth parasites have a remarkable ability to persist within their mammalian hosts, which is largely due to their secretion of molecules with immunomodulatory properties. Although the soluble components of helminth secretions have been extensively studied, the discovery that helminths release extracellular vesicles (EVs) has added further complexity to the host-parasite interaction. Whilst several studies have begun to characterise the molecules carried by helminth EVs, work aimed at investigating their biological functions has been hindered by a lack of helminth-specific EV markers. To begin to address this, we summarised helminth EV literature to date. With a focus on the protein and microRNA (miRNA) cargo, we aimed to detect similarities and differences across those major groups of helminths for which data are available; namely nematodes, trematodes and cestodes. Pfam analysis revealed that although there is no universal EV marker for all helminth species, the EF-hand protein family was present in all EV datasets from cestodes and trematodes, and could serve as a platyhelminth EV biomarker. In contrast, M13 metallopeptidases and actin may have potential as markers for nematode EVs. As with proteins, many miRNA families appeared to be species-, stage-, or dataset-specific. Two miRNA families were common to nematode EVs (mir-10 and let-7); the miRNA cargo of EVs secreted by clade I species appeared somewhat different from species from other clades. Five miRNA families (mir-71, mir-10, mir-190, let-7 and mir-2) were shared by all trematode species examined. Our analysis has identified novel markers that may be used in studies aimed at characterising helminth EVs and interrogating their function at the host-parasite interface. In addition, we discuss the heterogeneity of methods used for helminth EV isolation and emphasise the need for a standardised approach in reporting on helminth EV data.

1. Introduction

Parasitic helminths (worms) cause various diseases in humans, animals and plants. The prevalence of helminth infections in humans has been estimated at approximately 1.75 billion in 2016, resulting in 7 million years lived with disability (GBD 2016 Disease and Injury Incidence and Prevalence Collaborators, 2017). Infections are even more widespread in grazing ruminants: depending on the pathogen and region in western Europe, up to 80% of cattle and/or sheep herds were reported to be infected, impacting the meat and dairy industry (Charlier et al., 2014). Their success as pathogens is also reflected by their longevity in their hosts, with some species surviving for years and even decades in their hosts.

Helminths have evolved a plethora of strategies to survive and reproduce in exposed extracellular locations despite host defences. They may evade, modulate or even subvert each component of the

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https://doi.org/10.1016/j.ijpara.2020.04.010
0020-7519/

immune response that could be detrimental to them (Maizels and McSorley, 2016). All host-parasite associations have been shaped over time by an evolutionary arms race. While hosts have restricted the pathogens' deleterious impact, the latter have managed to establish and reproduce in spite of host responses. Hence, the constant crosstalk between hosts and parasites has led to the evolution of highly sophisticated relationships, characterised by complex molecular dialogues, where only precisely delimited conditions permit a successful infection.

In that context, much attention has been given to molecules released by parasites that may influence host immune responses. The composition of helminth excretory/secretory (E/S) products, referring to both the excreted 'waste' products and actively released 'functional' molecules, has been analysed in several species and assessed for their potential as diagnostic biomarkers of infection or vaccine targets (Harnett, 2014). In addition, there is considerable interest in E/S products for identification of the molecular and genomic adaptations underlying parasitism. The majority of these studies have focused on characterising the soluble protein fraction of E/S products due to methodological limitations and obvious functional roles, resulting in large catalogues of freely secreted E/S proteins. It is only recently that refined detection methods and technologies have allowed a broader picture of the helminth 'secretomes', beyond the protein compartment, encompassing lipid-based molecules, carbohydrates (glycoproteins and glycolipids), small metabolites, and nucleic acids to be obtained (van der Kleij et al., 2002; Prasanphanich et al., 2013; Claycomb et al., 2017; Hokke and van Diepen, 2017; Maizels et al., 2018; Wangchuk et al., 2019).

An additional layer of complexity was added with the discovery of extracellular vesicles (EVs) released by helminths (Marcilla et al., 2012). These nano-sized bodies, delimited by a lipid bilayer, have emerged as a ubiquitous vehicle for transferring information between cells, organisms and even across kingdoms of life (Woith et al., 2019). Distinct EV subtypes are often distinguished based on their subcellular origin and size: exosomes derive from the endocytic pathway and measure between 30 and 150 nm; microvesicles bud from the plasma membrane and range between 100 and 1000 nm, while apoptotic bodies can reach up to 5000 nm in diameter (van Niel et al., 2018). Secretion of EVs have been reported for all parasitic helminth groups, and their compelling roles in host-parasite interactions are being revealed (Eichenberger et al., 2018; Tritten and Geary, 2018; Zakeri et al., 2018). In addition to proteins, helminth-derived EVs are enriched in other putative effectors such as microRNAs (miRNAs), which can have important regulatory functions in gene expression by suppressing translation of targeted mRNA (Bartel, 2004). Furthermore, helminth-derived miRNAs have been detected in host biofluids and may represent novel biomarkers of infection (Hoy et al., 2014; Tritten et al., 2014; Cai et al., 2016; Hansen et al., 2016; Meningher et al., 2017; Liu et al., 2019; Zhou et al., 2019).

Although EVs appear to be instrumental in various and fundamental processes such as parasite survival and propagation, transfer of virulence factors, and modulation of host immunity (reviewed in Montaner et al, (2014); Coakley et al., 2015) the molecular mechanism(s) by which they achieve these effects have only been partially characterised (reviewed by Eichenberger et al. (2018b); Tritten and Geary (2018)). A more complete understanding of how helminth-derived EVs interact with, and modulate the effects of, host cells is dependent on further characterization of their molecular cargo molecules. Here, we summarise and discuss the current body of knowledge on helminth parasite EV molecular contents, with emphasis on protein and miRNA collections. We examined the methodologies employed by the various studies published to date and provide an initial comparison of unique and shared features across helminth phyla, and propose possible lines of action to shape future research.

2. Helminth EV datasets: species studied and methodologies applied

Following an extensive search of the literature, we identified 29 publications, describing 33 different datasets of helminth EVs and their cargo origin from 18 parasites (including 25 parasite life stages) (Table 1). Nine studies describing EV protein contents of six nematode species (*Ascaris suum* adults (Hansen et al., 2019), *Brugia malayi* L3 and adult males and females (Zamanian et al., 2015; Harischandra et al., 2018), *Heligmosomoides polygyrus* adults (Buck et al., 2014), *Nippostrongylus brasiliensis* adults (Eichenberger et al., 2018a), *Teladorsagia circumcincta* L4 (Tzelos et al., 2016), and *Trichuris muris* adults (Eichenberger et al., 2018; Shears et al., 2018; Tritten et al., 2017) were included. Eleven reports based on six trematode species were used (*Dicrocoelium dendriticum* (Bernal et al., 2014), *Echinostoma caproni* (Marcilla et al., 2012), *Fasciola hepatica* (Marcilla et al., 2012; Cwiklinski et al., 2015; Davis et al., 2019; de la Torre-Escudero et al., 2019), *Opisthorchis viverrini* (Chaiyadet et al., 2015), *Schistosoma japonicum* (Zhu et al., 2016a; Liu et al., 2019) and *Schistosoma mansoni* (Nowacki et al., 2015; Sotillo et al., 2016; Samoil et al., 2018)). Finally, we exploited EV data from seven reports based on five cestode parasites; *Echinococcus granulosus* (Siles-Lucas et al., 2017; Nicolao et al., 2019; Zhou et al., 2019), *Echinococcus multilocularis* (Zheng et al., 2017; Ding et al., 2019), *Mesocestoides corti* (Ancarola et al., 2017), *Taenia crassiceps* (Ancarola et al., 2017), and *Taenia asiatica* (Liang et al., 2019).

The EV-derived miRNAs were described in detail for six nematode (*A. suum* (Hansen et al., 2019), *B. malayi* (Zamanian et al., 2015), *H. polygyrus* (Buck et al., 2014), *N. brasiliensis* (Eichenberger et al., 2018a), *T. muris* (Eichenberger et al., 2018; Tritten et al., 2017), and *Haemonchus contortus* (Gu et al., 2017)), four trematode (*D. dendriticum* (Bernal et al., 2014), *F. hepatica* (Fromm et al., 2015), *S. mansoni* (Nowacki et al., 2015; Samoil et al., 2018), and *S. japonicum* (Zhu et al., 2016a; Liu et al., 2019) and three cestode species (*M. corti* (Ancarola et al., 2017), *T. crassiceps* (Ancarola et al., 2017), and *E. multilocularis* (Ding et al., 2019)). Furthermore, two further studies demonstrated the presence of EVs secreted from the nematodes *Trichinella spiralis* and *Trichuris suis* (including the presence of miRNA cargo), without further detailed descriptions of their cargo (Hansen et al., 2015; Kosanović et al., 2019).

The different publications report the use of various EV isolation and characterization methods. Among the various purification techniques described, ultracentrifugation (UC) as the sole EV enrichment method was used for 63.6% (21 of 33) of the datasets/helminth life stages. Similarly, data from EV studies on 41 different organisms collected in Vesiclepedia, a publically-available repository for vesicle cargo, indicated a predominance of EV enrichment using UC protocols with 57.7% of the 1254 included studies (Pathan et al., 2019). To reach a high purity of the enriched EV fractions, a community consensus guideline suggests to combine at least two different purification techniques or other verified vesicle purification methods (Théry et al., 2018). Accordingly for helminths, other techniques were used such as UC vesicle pre-enrichment followed with a secondary method in 18.2% of the records, such as the combination with density gradient purification (five of 33) and with a commercial kit (one of 33). Further techniques were the sole use of commercial kits (exoEasy Maxi Kit, ExoQuick-TC, miRCURY Exosome Isolation Kit, Total Exosome Isolation Kit) in 15.2% of the reports (five of 33), and a single size exclusion chromatography step in one study (3.0%).

Table 1
Summary of described helminth extracellular vesicle (EV) protein and microRNA (miRNA) contents.

Organism	Clade	Stages/Source	Number of worms/ volume	EV isolation method	PROTEIN			miRNA		Source data
					Number of proteins reported	Amount of protein used (μg)	Analysis method	Number of miRNAs reported	Analysis workflow	
NEMATODES										
Haemonchus contortus	V	Adults	100/ml	UC	–	–	–	40	Illumina GAIIx/LC Sciences workflow or miRDeep2	(Gu et al., 2017)
H. contortus	V	L4	250/ml	UC	–	–	–	37	Illumina GAIIx/LC Sciences workflow or miRDeep2	(Gu et al., 2017)
Heligmosomoides polygyrus	V	Adults	NA	UC	362 (139 specifically enriched in EVs)	5	LC-MS/MS	173 (high-confidence, not limited to EVs)	Illumina GAIIx/ miRDeep2/ mireap	(Buck et al., 2014)
Nippostrongylus brasiliensis	V	Adults	500/ml	UC + Optiprep (Iodixanol)	81	50	1D electrophoresis, LC-MS/MS	52	Illumina NextSeq 500/ miRDeep2	(Eichenberger et al., 2018a)
Teladorsagia circumcincta	V	L4	NA	UC	85	10	2D electrophoresis, LC-ESI–MS/MS	–	–	(Tzelos et al., 2016)
Ascaris suum	III	Adults	5/550 ml	UC	268	100	UPLC-nanoESI MS/MS	29	Illumina HiSeq 2500/ Bowtie mapping	(Hansen et al., 2019)
A. suum	III	L4	NA	UC	–	–	–	51	Illumina HiSeq 2500/ Bowtie mapping	(Hansen et al., 2019)
A. suum	III	L3	NA	UC	–	–	–	40	Illumina HiSeq 2500/ Bowtie mapping	(Hansen et al., 2019)
Brugia malayi	III	L3	300/ 25 ml (5000 total)	UC	31	15	1D electrophoresis, nano LC-MS/MS	52	Illumina miSeq v3/ miRDeep2	(Zamanian et al., 2015)
B. malayi	III	Adults	10 M/3 ml, 10 F/ 10 ml	UC	80	15	1D electrophoresis, nano LC-MS/MS	–	–	(Harischandra et al., 2018)
Trichuris muris	I	Adults	10–20/ml (3000 total)	ExoQuick TC	73	1	LC-MS/MS	14	Illumina/LC Sciences workflow	(Tritten et al., 2017)
T. muris	I	Adults	500/ 4.5 ml	UC + Optiprep (Iodixanol)	364	100	1D electrophoresis, LC-MS/MS	56	Illumina NextSeq 500/ miRDeep2	(Eichenberger et al., 2018c)
T. muris	I	Adults	NA	UC	125	NA	LC-MS/MS	–	–	(Shears et al., 2018)
TREMATODES										
Dicrocoelium dendriticum	NA	Adults	25/ml	UC	84	NA	LC-MS/MS	42	Applied Biosystems 5500xl Genetic Analyzer/ miRanalyzer	(Bernal et al., 2014)
Echinostoma caproni	NA	Adults	2/ml	UC	81	NA	LC-MS/MS	–	–	(Marcilla et al., 2012)
Fasciola hepatica	NA	Adults	10/ml	UC	98	NA	LC-MS/MS	55	Applied Biosystems 5500xl Genetic Analyzer/ miRCandRef/ miRDeep2/ miRanalyzer	(Marcilla et al., 2012; Fromm et al., 2015)

(*continued on next page*)

Table 1 (*continued*)

Organism	Clade	Stages/Source	Number of worms/ volume	EV isolation method	PROTEIN			miRNA		Source data
					Number of proteins reported	Amount of protein used (μg)	Analysis method	Number of miRNAs reported	Analysis workflow	
F. hepatica	NA	Adults	2/ml	UC	180	NA	LC-MS/MS	–	–	(Cwiklinski et al., 2015)
F. hepatica	NA	Adults	1/ml	UC	380 (EV surface)	NA	Biotinylation and LC-MS/MS	–	–	(de la Torre-Escudero et al., 2019)
F. hepatica	NA	Adults	1/ml	UC or SEC	392 (UC) and 321 (SEC)	NA	1D electrophoresis, LC-MS/MS	–	–	(Davis et al., 2019)
Opisthorchis viverrini	NA	Adults	NA	UC	108	NA	LC-MS/MS	–	–	(Chaiyadet et al., 2015)
Schistosoma japonicum	NA	Juvenile (liver stage)	10/ml	Total exosome isolation kit	403	NA	1D electrophoresis, LC-MS/MS	35	Illumina HiSeq 2000/ Mireap	(Zhu et al., 2016a)
S. japonicum	NA	Adults	NA	UC + Total exosome isolation kit	–	–	–	38	Illumina HiSeq 2000/ Bowtie	(Liu et al., 2019)
Schistosoma mansoni	NA	Schistosomula	7500/ml	UC	109	NA	LC-MS/MS	167	Illumina HiSeq/ miRDeep2	(Nowacki et al., 2015)
S. mansoni	NA	Adults	NA	UC + Optiprep (Iodixanol)	83	2	1D electrophoresis, LC-MS/MS	–	Illumina GAIIx/LC Sciences workflow	(Sotillo et al., 2016)
S. mansoni	NA	Adults	2/ml	UC + Sucrose gradient	130	200	LC-MS/MS	143	Illumina GAIIx/LC Sciences workflow	(Samoil et al., 2018)
CESTODES										
Echinococcus granulosus s.l.	NA	Hydatid fluid from fertile cysts (Metacestode)	200 ml	UC + Optiprep (Iodixanol)	663	20	1D electrophoresis, LC-MS/MS	–	–	(Siles-Lucas et al., 2017)
E. granulosus s.l.	NA	Protoscolex	9000 total	UC	112	100	1D electrophoresis, LC-MS/MS	–	–	(Nicolao et al., 2019)
E. granulosus s.l.	NA	Hydatid fluid from fertile cysts (Metacestode)	NA	exoEasy Maxi Kit	936	NA	LC-MS/MS	–	–	(Zhou et al., 2019)
E. granulosus s.l.	NA	Protoscolex	3000 PS/ well	exoEasy Maxi Kit	149	NA	LC-MS/MS	–	–	(Zhou et al., 2019)
Echinococcus multilocularis	NA	Metacestode	NA	miRCURY Exosome Isolation Kit	433	100	nano LC-MS/MS	18	Illumina HiSeq/ miRDeep	(Zheng et al., 2017; Ding et al., 2019)
Mesocestoides corti	NA	Tetrathyridium	20–40 μl/ well × 12	UC	64	NA	LC-MS/MS	1	RT-PCR	(Ancarola et al., 2017)
Taenia asiatica	NA	Adult	1/50 ml	UC	455	100	LC-MS/MS	NA	Illumina HiSeq 2500/ Mireap	(Liang et al., 2019)
Taenia crassiceps	NA	Cysticercus	10–15 ml/ flask	UC	195	NA	LC-MS/MS	7	RT-PCR	(Ancarola et al., 2017)

NA, not available or not reported; UC, ultracentrifugation; RT, reverse transcription.
ExoQuick-TC: System Biosciences, USA; Total exosome isolation kit: Thermo Fisher, USA; ExoEasy: Qiagen, Germany; miRCURY Exosome Isolation Kit: Exiqon, Germany.

Protein and miRNA contents of helminth-released EVs are summarised in Table 1. While the majority of reports focussed on the adult stage of the different helminths studied, other stages (i.e. larvae, metacestodes, schistosomula, tetrathyridium) had been also studied and are indicated.

The presence of EVs in the various studies was demonstrated mostly by transmission electron microscopy (TEM). If TEM was not performed, nanoparticle size distribution was measured by Tunable Resistive Pulse Sensing technology (TRPS; qNano®; IZON) or Nanoparticle Tracking Analysis (NTA; e.g., NanoSight®; Malvern Panalytical). Further, evidence for EVs was indicated by mass spectrometry or western blot to confirm the presence of proteins homologous to those commonly detected in mammalian EVs. Thereby, proteomics studies on helminth EVs detected several such parasite protein homologues (see Section 3). Consequently, immunoblotting analyses have been used to present selected helminth EV proteins, mainly in trematodes. Immune sera against recombinant versions demonstrated peroxiredoxin, cathepsin L1, FABP-V, DM9 domain-containing proteins, myoferlin, alix, acid sphingomyelinase, TSG101, ral-A, tetraspanin CD63 domains, GST and leucine aminopeptidase in *F. hepatica* EVs (Cwiklinski et al., 2015; Davis et al., 2019; de la Torre-Escudero et al., 2019), heat

shock protein 90 (HSP90) in *S. japonicum* EVs (Liu et al., 2019), and TSP-2 in *S. mansoni* EVs (Sotillo et al., 2016). Further, cross-reacting antibodies were used to demonstrate EVs by anti-*S. mansoni* leucine aminopeptidase in *F. hepatica*, by anti-mammalian GAPDH and HSP70 in *S. japonicum*, and by anti-human enolase in *S. mansoni* (Cwiklinski et al., 2015; Samoil et al., 2018; Liu et al., 2019). These analyses, however, show that some proteins are more abundant in EVs than others. As an example, it was shown for the cestode *E. multilocularis* that antibodies against recombinant parasite 14-3-3 highly reacted against EV blots compared with metacestode membrane fractions, whereas anti-enolase antibodies did not show much differential expression (Zheng et al., 2017). However, such comparisons of antibody reaction to different parasite fractions and evaluation of a subsequent specific detection of EVs are generally missing. The demonstration of parasite vesicles isolated from host tissue, from parasites co-cultured with feeder cells or requiring serum-supplemented media, by using antibodies against homologue proteins known to be present in mammalian EVs, appears especially challenging. For example, for *E. granulosus* samples isolated from patients, the presence of EVs was demonstrated based on anti-human CD9 and anti-CD63 (tetraspanins with homology of 21–30%) (Nicolao et al., 2019; Zhou et al., 2019), while other studies used anti-human TSPAN14 (another tetraspanin) to demonstrate host contamination (Siles-Lucas et al., 2017).

3. Proteomic datasets of helminth EV cargo

Helminth EVs have been identified as an important component in the host-parasite interaction. Although the mechanistic understanding of the EV contribution to this interaction is still largely unknown, it is likely that key effector molecules involved include active proteins contained in the EVs. To this end, most studies in the field to date have focused on characterising the protein component of helminth EVs (see Table 1 and Supplementary Tables S1-S3). The published EV protein sequences used in this work are available at Mendeley Data, DOI: 10.17632/x3xvvz395z.2. The number of proteins identified in each study was variable, likely reflecting different sample collection conditions, EV purification protocols and detection methods (e.g. mass spectrometry approaches) used across the studies.

Putative functions of discrete EV proteins in helminth-host crosstalk, immune regulation, and in promoting pathogenesis have been discussed in a number of recent review articles (Eichenberger et al., 2018; Suttiprapa et al., 2018; Tritten and Geary, 2018; Wu et al., 2019; Zakeri et al., 2018). However, a systematic comparison of different helminth species is currently lacking. To address conserved and unique features across phyla and species, an initial Pfam family and domain classification of all available proteomic datasets was performed (see Supplementary Methods S1). The number of unique Pfam accessions approximately reflected the number of proteins in the dataset identified by each study, and the majority of these unique Pfam accessions appeared to be species-specific (Fig. 1 and Supplementary Tables S1-S3).

For nematodes, only three Pfam identifiers were common to all six species (although not to all datasets): PF05649.13, PF01431.21 (both M13 metallopeptidases), and PF00022.19 (actin). Gastrointestinal nematodes (all species but *B. malayi*) showed two items in common: PF01060.23 (transthyretin-like family proteins) and PF00026.23 (aspartic protease). There was, a priori, no clear clustering according to nematode phylogenetic clades. Six Pfam accessions were common to all six trematode species examined (although not to all datasets): PF00091.25 and PF03953.17 (tubulins), PF00012.20 (heat-shock protein 70), PF00240.23 (ubiquitin), PF00022.19 (actin), and PF13499.6 (EF-hand). The cestodes show, in general, larger datasets, and as many as 44 Pfam accessions

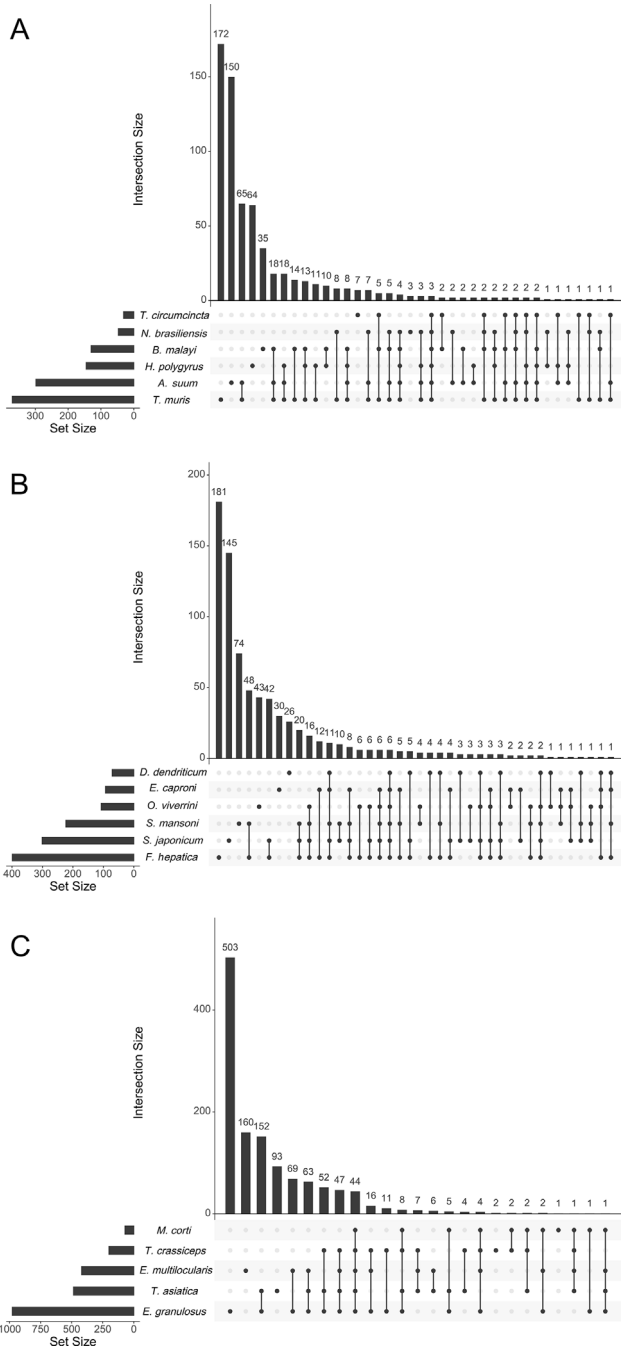

Fig. 1. Unique Pfam accessions are largely dataset-specific. UpSetR analysis visualising intersections between unique Pfam accessions found in the extracellular vesicle (EV) proteomes of six nematode (A), six trematode (B), and five cestode (C) species. Intersecting datasets are shown as filled circles; the corresponding number of unique Pfam accessions in that intersection is presented on top of each histogram. The set size represents the number of unique Pfam accessions found in each dataset. For a complete list of Pfam names see Supplementary Tables S1-S3.

overlapped across all five examined cestode species. Furthermore, 69 accessions were common and unique to *Echinococcus* spp., while four were common and unique to *Taenia* spp.

Additionally, the curation of available datasets allowed for the analysis of the abundance of particular domains in the different datasets with the goal of identifying specific proteins and protein families that could serve as bona fide markers of trematode, nematode or cestode EVs (Supplementary Tables S1-S3). In general, the most abundant protein families differed between trematodes,

nematodes and cestodes, although these families seemed to be specific for parasite species or species with similar life cycles. For instance, some of the most abundant families in trematodes were Ras (PF00071) and Peptidase_C1 (PF00112), particularly in *F. hepatica* datasets and the *S. japonicum* dataset from Zhu et al. (Cwiklinski et al., 2015; Zhu et al., 2016a; Davis et al., 2019; de la Torre-Escudero et al., 2019). The only family of proteins that was present in all trematode datasets was EF-hand (PF13499), while traditional exosome markers such as proteins belonging to the tetraspanin family (PF00335), although abundant in *Schistosoma* spp. datasets and several *F. hepatica* datasets, were not present in the *E. caproni*, *D. dendriticum* and one of the *F. hepatica* datasets, most likely due to methodological reasons (Supplementary Table S1). Interestingly, proteins belonging to the Tubulin and Tubulin_C families (PF00091 and PF03953, respectively) were more represented in datasets of EVs secreted by liver flukes (Bernal et al., 2014; Chaiyadet et al., 2015; Davis et al., 2019). In nematodes, proteins from the Trypsin family (PF00089) were highly abundant in all three *T. muris* datasets (Eichenberger et al., 2018; Shears et al., 2018; Tritten et al., 2017) (Fig. 1A, Supplementary Table S2). Additionally, proteins belonging to the Ras family (PF00071) were also highly abundant in *H. polygyrus*, *A. suum* and *B. malayi* (Buck et al., 2014; Harischandra et al., 2018; Hansen et al., 2019), although those were absent in the *N. brasiliensis* and two of the *T. muris* datasets (Eichenberger et al., 2018; Tritten et al., 2017). Interestingly, and similar to what was observed in trematodes, some of the most abundant families (that were also present in all datasets) were Ras (PF00071), EF-hand (PF13499), Tubulin (PF00091) and Tubulin_C (PF03953). Other highly abundant families and domains present in all datasets were the C2 domain (PF00168) and Dynein_light (PF01221). Pfam accessions were very heterogenous in cestodes; the Ras family (PF00071) was abundant across all datasets. The most abundant protein profile was different in *E. granulosus* hydatid fluid (Zhou et al., 2019) compared with the other datasets, with Dynein (PF01221) and Cadherin (PF00028) occupying top ranks of abundance.

Although the datasets are highly diverse, this analysis showed some protein families and domains, commonly found in EVs, which could serve as markers, such as tetraspanin family proteins, structural proteins (actin, dynein, tubulin, C2 domain containing proteins), and proteins involved in EV biogenesis (Ras and Rab). It seems that there is no collective marker for all helminth EVs, but the presence of a sub-set of common proteins, however, can be indicative of an EV biomarker, depending on the species and phyla. For example, the EF-hand family was present in all EV datasets from the cestodes and trematodes, and could serve as a potential platyhelminth EV biomarker. In addition, M13 metallopeptidases and actin seem to be good potential markers for nematode EVs. Also, previous reports found proteins belonging to the SCP/TAPS (SCP/Tpx-1/Ag5/PR-1/Sc7) family (PF00188) common in EVs from several nematode species. The Pfam domains and families common to all datasets analysed from each lineage (trematodes, nematodes and cestodes) are schematically illustrated in Fig. 2. Although not evident in our Pfam analysis, other proteins generally detected in eukaryotic EVs could be found frequently in helminth EV datasets, including 14-3-3, and heat shock proteins (Hsp60, Hsp70, Hsp90/HTpG, and DnaJ/Hsp40), and should be considered in the discovery of proteomic EV markers, some of which are more abundant in different EV classes (summarised in Mekonnen et al. (2018) and Wu et al. (2019)).

It should be noted that the proteomic cargo may not just reflect the internal 'cytosolic' proteins but also the structural proteins integral to the membrane, and furthermore, proteins associated on the outer surface of vesicles. Transmembrane proteins with and without outer domains could be predicted based on various available algorithms (Möller et al., 2001). For example, for *F. hepatica* EVs, surface-exposed proteins have been studied by biotin-labelling and enzymatic shaving of EVs, followed by a pull-down proteomics approach, identifying 380 proteins potentially involved in the molecular interaction between EVs and host cells (de la Torre-Escudero et al., 2019). Minimal information is available for other helminth species, although it is worth distinguishing the sub-vesicular location of studied EV proteins, which could present direct functional consequences.

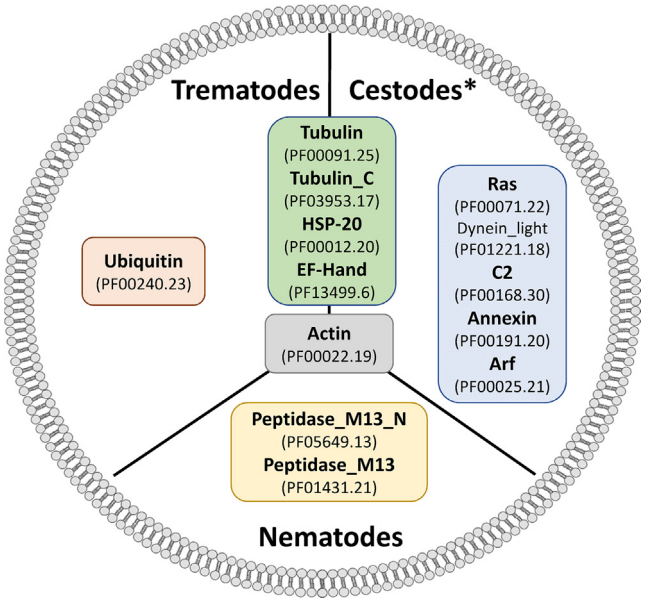

Fig. 2. Schematic representation of Pfam domains and families common to all datasets analysed from each lineage (trematodes, nematodes and cestodes). *For cestodes, only the top 10 Pfam domains/families based on occurrence are shown for clarity. For a complete list of Pfam names see Supplementary Tables S1-S3.

4. The miRNA cargo of helminth EVs

EVs have been shown to be a vehicle for the transport of genetic material as a form of cell-to-cell communication (Raposo and Stoorvogel, 2013) and nucleic acids are a ubiquitous constituent of EV cargo. Pathogens take advantage of EVs for cross-species and cross-kingdom communication, exploiting these natural cell-to-cell communication pathways in order to modulate host gene expression (Coakley et al., 2015; Cai et al., 2019). In the helminth-host interaction, most of the focus to date has been on miRNAs, a conserved class of small non-coding RNA in animals. We collected available datasets on miRNAs described from parasitic helminths EVs (Table 1). Similar to the proteomic datasets, great diversity in the approaches and methodologies used for miRNA sequencing or detection was observed. Similarly, the reporting of information generated by these efforts varied substantially across studies. For instance, some reported annotations without sequences, some focused on the top 20 miRNAs without describing the less abundant members, and some reported sequences amplified in targeted quantitative PCR (qPCR) experiments (Table 1, Supplementary Table S4). Our raw analysis was based on the assigned families for the 20 most abundant miRNAs reported in each study, mostly based on miRNA annotations and family information, some of which were re-annotated (see Supplementary Methods S1). Again, as for proteins, many miRNA families identified in helminth EVs appeared to be species- or dataset-specific (as defined by the top 20 miRNAs exclusively).

Two miRNA families were commonly found in all nematode species (mir-10 and let-7; Fig. 3). Mir-71 and lin-4 family members

were present in all nematodes except the clade I species *T. muris*. All four trematodes species analysed shared mir-71, mir-10, mir-190, let-7 and mir-2 among the most abundant miRNA families. The mir-9 family appeared to be absent among the 20 most abundant EV miRNAs of trematodes (but was present in datasets from nematode clade I and III species, and from the cestode *T. asiatica*). Thirty-nine of these most abundant miRNA families appeared to be uniquely associated with nematode EVs, 13 with trematode EVs, and five with cestode EVs (Fig. 3, Supplementary Table S4).

Functionally, the miRNA host transcript regulation has mainly been studied with respect to immune modulation and linked to parasite survival. The effects of individual miRNAs are discussed in recent review articles (Arora et al., 2017; Cai et al., 2019; Eichenberger et al., 2018; Fromm et al., 2017; Tritten and Geary,

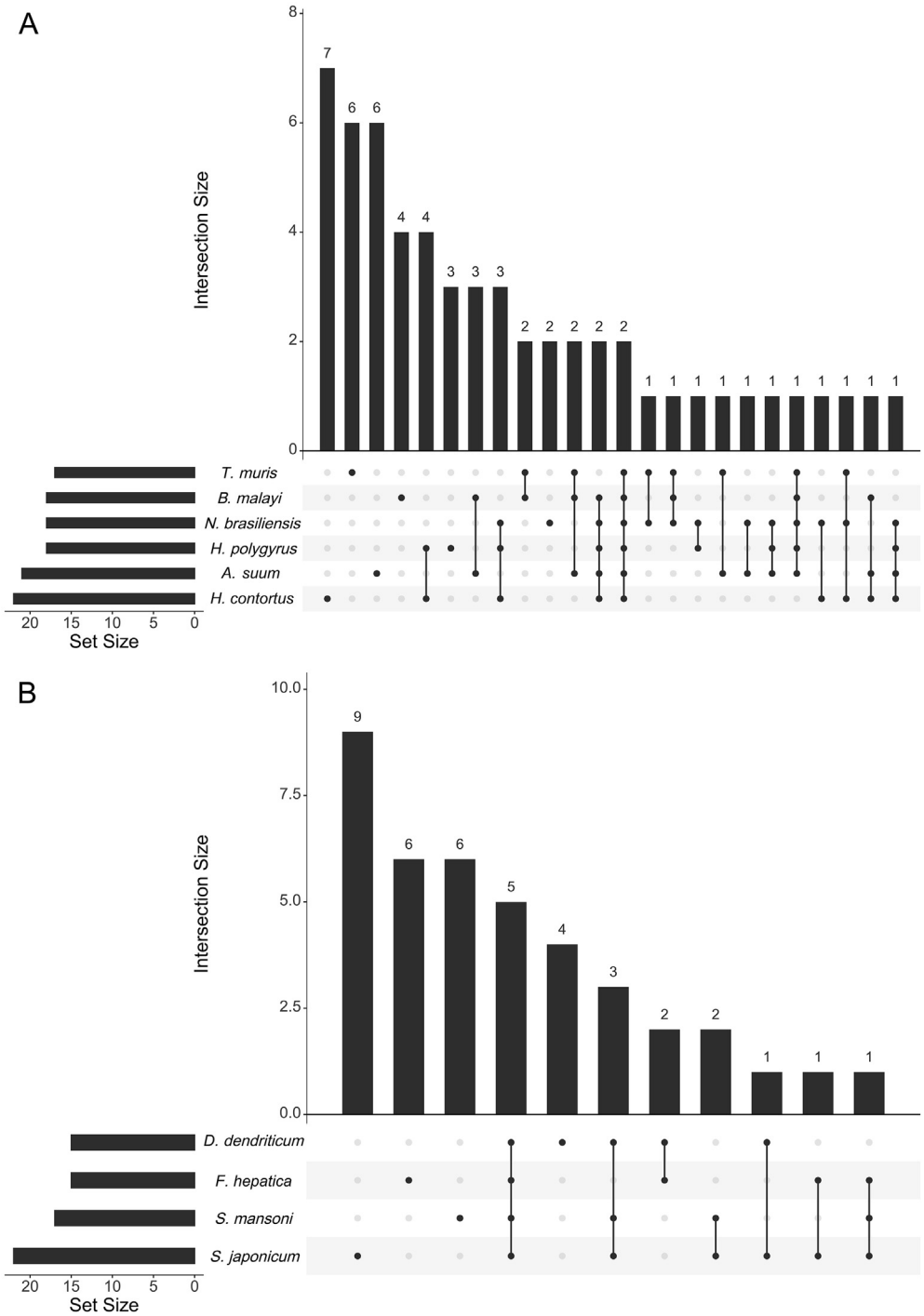

Fig. 3. Unique microRNA (miRNA) families. UpSetR analysis visualising intersections between unique miRNA families among the 20 most abundant miRNAs found in extracellular vesicles (EVs) of six nematode (A), and four trematode (B) species (all stages confounded). Intersecting datasets are shown as filled circles; the corresponding number of unique miRNA families in that intersection is presented on top of each histogram. The set size represents the number of unique miRNA families found in each dataset. miRNA data from cestodes was not included here, as it does not result from sequencing experiments and represents very small datasets. The list of the top 20 miRNAs used here can be found in Supplementary Table S4.

2018). Interestingly, the E/S products of the carcinogenic liver fluke *Clonorchis sinensis* induces a dysregulation of known cancer-associated miRNAs with important roles in pathological processes, mainly involved in the generation of biliary fibrosis associated with the parasite (Pak et al., 2014). Whether carcinogenic parasites induce fibrosis by active EV-mediated interaction in miRNA-regulated signalling pathways in host tissues has yet to be discovered. Furthermore, miRNAs (not restricted to EVs) are not only involved in inter-individual communication (and across species): they have been extensively studied for their important functions in the regulation of the worm's reproduction and development, longevity and physiology, both in the free-living nematode *Caenorhabditis elegans* and parasitic species (Lim et al., 2003; Winter et al., 2012, 2015; Zhu et al., 2016b; Marks et al., 2019; Pérez et al., 2019).

Further, non-coding RNA species have been reported as integral parts of helminth EVs. *Heligmosomoides polygyrus* EVs were shown to contain Y RNA and siRNA, while those derived from *S. mansoni* carried tRNA-derived small RNA (tsRNA) (Buck et al., 2014; Nowacki et al., 2015; Chow et al., 2019). Functionally, roles for these other kinds of non-coding RNAs in cross-species communication remain to be fully characterised. However, there is accumulating evidence showing their likely importance in these exchanges (Cai et al., 2019). Another cargo of helminth EVs is mRNA. To date, mRNA EV cargo only has been studied in the clade I nematode *T. muris* (Eichenberger et al., 2018c). EV-packed mRNAs were mainly coding for proteins showing domains of unknown function, followed by sequences involved in reverse transcription and retrotransposon activity, suggesting a potential involvement of these mRNAs in direct interactions with genes and the genome. So far, there is no evidence of translation of helminth EV-derived mRNA in host cells. However, such an EV-driven mRNA transfer and host cell translation by a virus-like machinery would be consistent with the recurring motif that parasitic helminths adopt diverse strategies to hijack host physiology.

5. Missing data, and research priorities

In this analysis, we gathered datasets on EV molecular cargo from different parasitic helminth species and provided an initial analysis based on shared and unique domains and sequences. Comparing data across studies requires caution due to differences inherent to the studied organisms (biomass, developmental stage, etc.) as well as methodological and analytical aspects. The existing literature reports distinct EV isolation methods, which are known to impact on the quality and depth of the results. As an example, polymer-based precipitation methods (such as ExoQuick TC) warrant high recovery but low specificity; differential ultracentrifugation yields mixed EV populations and free protein aggregates (intermediate recovery and specificity) (Théry et al., 2018). Similarly, it is expected that some material is lost when different techniques are combined and an electrophoresis step is added before protein characterization by mass spectrometry. However, to report on EV cargo, particularly pure EV fractions are needed, which is not always provided by an enrichment method based on a single physical separation technique (Théry et al., 2018). A number of other techniques exist such as size exclusion chromatography, tangential flow filtration, field-flow fractionation, field-free viscoelastic flow, alternating current electrophoretics, acoustics, ion exchange chromatography microfiltration, fluorescence-activated cell sorting (FACS), deterministic lateral displacement arrays, immunoisolation or other affinity isolation technologies (including lipid affinity purification), precipitation/combination techniques, hydrostatic filtration dialysis, fast protein liquid chromatography (FPLC) and HPLC techniques, and a wide variety of microfluidics devices are suitable for helminth EV purification. The applicability of some of these alternatives for helminth EV purification has yet to be demonstrated.

However, standardised purity control, e.g. by a measure of nano-vesicle counts to protein concentration ratio (Webber and Clayton, 2013), or quality control by calculation of a score based on different experimental parameters as proposed by the EV-TRACK consortium (EV-TRACK Consortium, 2017) is recommended. This score (EV-METRIC) is based on applied separation and purification methods, protein analysis, and particle analysis. The EV-METRIC is already included as a quality check for data submission to the Vesiclepedia repository (Pathan et al., 2019). As this score compares vesicles across all three kingdoms of life, its value for helminth EVs has to be further investigated.

Based on the diverse sets of annotated data, our preliminary approach to compare helminth species did provide some hints of species- or phylum-specific protein and abundant (based on top 20 only) miRNA EV markers, and these should be considered in future helminth EV characterization efforts. It is clear, however, that a standardised reporting of data on helminth EV cargo would improve and facilitate future research, by allowing direct comparison between datasets and help identify functional commonalities between species. Consequently, a regular revision of miRNA annotation by name and family attributes needs to be undertaken. A recent miRNA curation work revealed that miRBase entries are often inaccurately annotated or may not represent bona fide miRNAs, which led to the initiation of an alternative repository, MirGeneDB 2.0 (Fromm et al., 2020). This platform currently contains 45 species across the most important metazoan groups; entries are selected based on the identification of miRNA genes and families rather than naked sequences. At this stage, the platform includes three nematodes (two *Caenorhabditis* spp. and *A. suum*), and hence, requires population with more helminth data to enable comparative genomic approaches in helminth miRNA research.

Although helminth EV research is still in its infancy, there is a growing number of reports on EVs and their cargo. To keep track of these, current and future EV datasets should be gathered in a public repository, increasing data visibility and availability. Different compendia for vesicles and their cargo are accessible online; ExoCarta for exosome cargo (Keerthikumar et al., 2016), EVpedia for high-throughput datasets from vesicles – currently not available (Kim et al., 2015), and Vesiclepedia representing a community-driven repository of EV cargo with EV-METRIC based quality control (evtrack.org), already includes helminth EV datasets (Pathan et al., 2019). We emphasise – as a critically important point – to make raw sequencing data publicly available, allowing for re-annotation and quality control, to support continuous improvement of the curation and annotation of helminth data.

Acknowledgments

JS is a Miguel Servet Fellow funded by Instituto de Salud Carlos III, Spain (CP17III/00002). AM is supported by the Conselleria d'Educació, Cultura I Esports, Generalitat Valenciana, Valencia, Spain (PROMETEO/2020/071) and by the Agencia Estatal de Investigación, Ministerio de Ciencia e Innovación (PID2019-105713GB-I00/AEI/10.13039/501100011033). MR is supported by a grant (BB/L019612/1) from the Biotechnology and Biological Sciences Research Council, UK (BBSRC). MK is supported by an R21 award (AI117204) from the National Institute of Allergy and Infectious Diseases, USA (www.niaid.nih.gov). MC and MEA are supported by Perez Guerrero Trust Fund, ANPCyT 2017N°2062 and National Scientific and Technical Research Council (CONICET), Argentina. PN is supported by the Independent Research Fund Denmark (DFF-6111-00521). LT and RME are supported by the Swiss National Science Foundation (PZ00P3_168080 and

PZ00P3_185865). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpara.2020.04.010.

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