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Original Article

The core exosome proteome of *Trichomonas vaginalis*

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KEYWORDS
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Abstract *Background: Trichomonas vaginalis* is parasitic protozoan that causes human urogenital infections. Accumulated reports indicated that exosomes released by this parasite play a crucial role in transmitting information and substances between cells during host-parasite interactions. Current knowledge on the protein contents in *T. vaginalis* exosome is mainly generated from three previous studies that used different *T. vaginalis* isolates as an experimental model. Whether *T. vaginalis* exosomes comprise a common set of proteins (core exosome proteome) is still unclear.
Methods: To explore the core exosome proteome in *T. vaginalis*, we used liquid chromatography-tandem mass spectrometry (LC-MS/MS) to identify the contents of sucrose ultracentrifugation-enriched exosome and supernatant fractions isolated from six isolates.
Results: Transmission electron microscopy (TEM) confirmed the presence of exosomes in the enriched fraction. Proteomic analysis identified a total of 1870 proteins from exosomal

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extracts. There were 1207 exosomal-specific proteins after excluding 436 'non-core exosomal proteins'. Among these, 72 common exosomal-specific proteins were expressed in all six isolates. Compared with three published *T. vaginalis* exosome proteome datasets, we identified 16 core exosomal-specific proteins. These core exosomal-specific proteins included tetraspanin (TvTSP1), the classical exosome marker, and proteins mainly involved in catalytic activity and binding such as ribosomal proteins, ras-associated binding (Rab) proteins, and heterotrimeric G proteins.

Conclusions: Our study highlighted the importance of using supernatant fraction from exosomal extract as a control to eliminate 'non-core exosomal proteins'. We compiled a reference core exosome proteome of *T. vaginalis*, which is essential for developing a fundamental understanding of exosome-mediated cell communication and host-parasite interaction.

Introduction

Exosomes are a specific subclass of extracellular vesicles (EV) that originate from endosomes by the invagination of the endosomal membrane and are then released into the extracellular space. They were first discovered in reticulocyte secretion in the 1980s.[1,2] These cup-shaped exosomes range in size from 30 to 150 nm, and are secreted by almost all cell types studied.[3,4] Exosomes are enveloped by a lipid bilayer membrane that incorporates various cell-specific components, including proteins, lipids, metabolites, and various nucleic acids such as DNA, mRNA, microRNA, and long noncoding RNAs. This composition partially mirrors the phenotype of the cells from which the exosomes originate. These exosomal cargoes are involved in cell-to-cell communication and the transfer of functional biomolecules between cells.[5] Moreover, exosome has been considered an important player in immune responses, cell proliferation, neuronal signaling, disease pathogenesis process, and as potential treatments for autoimmune disorders.[6,7]

Trichomonas vaginalis is a sexually transmitted protozoan parasite that can cause symptoms like vaginal discharge, itching, and pain in females. It can also infect men with milder symptoms. Several works have reported on the exosomes or EVs released from *T. vaginalis* in recent decades. Twu et al.[8] confirmed that *T. vaginalis* can modulate the host cells' physical and biochemical properties via secreting extracellular vesicles or exosomes for the first time. In addition, they demonstrated that *T. vaginalis* exosomes have a role in promoting parasite-parasite communication and host cell colonization.[8]

Previous studies on *T. vaginalis* exosomes have shown that exosomal cargo contains various molecules, including proteins, lipids, and RNA. These molecules are believed to play a role in the pathogenesis of *T. vaginalis* infections by carrying virulence factors and other molecules that facilitate the establishment of infection. *T. vaginalis*-derived exosomes can induce changes in the host cell and mediate the parasite's interaction with the host by increasing the adherence of the parasite to host cells.[8] Besides, *T. vaginalis* exosomes fuse with the host cell membrane, increase parasite adherence, and deliver their content (proteins, RNA, or lipids) to the host cells.[8,9] Lately, Rai and Johnson[9] also presented that *T. vaginalis* EVs interact with cell membranes and transfer cargo through lipid raft-mediated endocytosis. In addition, Olmos-Ortis et al.[10] suggested that exosome-like vesicles from *T. vaginalis* have an immunomodulatory role on cytokine profiles induced by the parasite and promote a decrease in the inflammatory process.

Proteomics analysis has been performed to define the protein content of different EV populations and further investigate the host-parasite interaction. Many molecules identified in *T. vaginalis* EV proteomes are also commonly found in EVs from other organisms. These proteins include ESCRT (endosomal complexes required for transport; VPS32)[11]; Rab GTP-ases; SNARE-complex proteins[12,13]; cytoskeletal proteins (actin, tubulin)[14]; metabolic enzymes (malic enzyme, glyceraldehyde 3-phosphate dehydrogenase); ribosomal proteins and tetraspanins: TvTSP1 in exosomes[8] and TvTSP8 in microvesicles.[15] In addition, a series of proteins related to pathogenesis has been detected, such as GP63 protease,[16] BspA proteins,[17] the surface immunogen protein p270,[18] TvMIF protein[19] and hypothetical proteins.[20] These proteins could indicate a role in establishing infection and modulating host immune responses.

Moreover, *T. vaginalis* EVs were found to encapsulate a cargo of small-size RNAs, particularly tRNA-derived small RNA (tsRNA).[8,21,22] These small RNAs derived from ribosomal rRNAs and tRNAs may contribute to the modulation of the target cell's gene expression.[23] Twu et al.[8] found a heterogeneous population of small RNAs ranging between 25 and 200 nt in *T. vaginalis* exosomes, which may play a critical role in modulating interactions between parasites, or parasites and hosts. On the other hand, recent reports also demonstrated that *Trichomonasvirus* (TVV) in exosomes released by *T. vaginalis* can also modulate the host response.[22,24] Rada et al.[22] proved that releasing TVV virions and associated components in small extracellular vesicle (sEV) cargo to the environment may reveal an increased risk for inflammation-related pathogenesis during Trichomoniasis.

Although it is well established that the exosomal cargo from *T. vaginalis* can modulate parasite-host interaction, the components of *T. vaginalis* exosomes reported from different studies vary greatly.[8,22,25] It is still unclear

whether the exosomal cargo results from a random process in the cytoplasm or involves a complex sorting mechanism that favors specific biomolecules over others. Also, the analysis of exosomes from *T. vaginalis* remains a technical challenge due to the contamination of the exosome proteome with proteins in the supernatant fraction. Therefore, profiling exosomal molecules from multiple *T. vaginalis* isolates is essential to answer this question. In the present study, we elucidated the core exosome proteome from six *T. vaginalis* isolates and established a standard protocol to remove 'non-core exosomal proteins' in the exosome proteome. These six isolates included ATCC 30236, 30238, 50148, 50143, PRA-98, and T1. All these isolates are sensitive to metronidazole except that ATCC 30238 and 50143 are resistant. In addition, all the isolates are positive for Trichomonasvirus (TVV) except ATCC 50143, which is free of TVV.

Methods

Trichomonas vaginalis culture

Trichomonas vaginalis isolates of ATCC 30236, 30238, 50148, 50143, PRA-98, and T1 were cultured at 37 °C in yeast extract, iron-serum (YI-S) medium containing 80 μM ferrous ammonium citrate (Sigma-Aldrich, St. Loius, Mo, USA).[26] The cells for assays were harvested from the mid-log phase. The trypan blue exclusion assay was used to count the number of viable cells.

Isolation of exosomes

The exosomes were isolated following the method from Gupta et al.[27] with some modifications.[22,24] One liter of each *T. vaginalis* culture at a density of approximately 2×10^6 cells/mL was harvested by centrifugation. The harvested cells were washed three times with 1 × PBS (pH 6), resuspended in YIS media without serum, and incubated at 37 °C for 4 h. Cells were removed by centrifugation at 2000×*g* for 15 min followed by centrifugation at 10,000×*g* for 30 min. The cell-free media was filtered through a 0.22 μm filter and concentrated using 10 kDa MWCO mPES hollow fiber D02 (Lefo Science Co., Ltd., Taipei, Taiwan) to approximately 10 mL. The filtered cell-free media were further concentrated through an Amicon Ultra15 centrifugal filter unit (MWCO 10 kDa; Millipore Corporation, Bedford, MA, USA) to a final volume of approximately 3.5 mL. The concentrated cell-free medium was loaded slowly over 0.5 mL of 30% sucrose solution and ultracentrifuged at 100,000×*g* for 2 h at 4 °C using a swinging bucket rotor (P56ST; Hitachi Koki Co., Ltd., Minato, Tokyo, Japan). The supernatant was kept as a control for proteomic analysis to determine the potential 'non-core exosomal proteins'. The sucrose layer was resuspended in 1 × PBS (pH 7) and ultracentrifuged again at 100,000×*g* for 2 h at 4 °C to pellet the exosomes. The isolated exosomes were resuspended in approximately 20 μL of 1 × PBS buffer (pH 7) and stored at −20 °C for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. The protein concentration of each exosomal sample was detected by Bio-Rad Protein Assay Dye Reagent Concentrate.

Transmission electron microscopy (TEM)

An aliquot (3 μL) for each exosome solution was coated onto a glow-charged formvar carbon grid. Briefly, the exosomal samples were fixed with 1% uranyl acetate. Then, the grids were air-dried and visualized using a JEM-2100 PLUS of TEM (JEOL, Tokyo, Japan) at 100 kV.

Proteomics data analysis

For protein identification, 5 μg of enriched exosomal protein and supernatant fractions from each preparation were sent to the Molecular Medicine Research Center at Chang Gung University. The proteins were subjected to in-solution tryptic digestion before LC-MS/MS analysis.[28] Each peptide sample was reconstituted with 0.1% formic acid (FA), and then analyzed by a nano-LC-LTQ-Orbitrap Hybrid Mass Spectrometer (Thermo Fisher, San Jose, CA, USA), as previously described.[29] Briefly, the sample was loaded across a trap column (Zorbax 300SB-C18, 0.3 × 5 mm; Agilent Technologies, Wilmington, DE, USA) at a flow rate of 0.2 μl/min in HPLC buffer (0.1% FA), and separated on a resolving 10-cm analytical C18 column (inner diameter, 75 μm) using a 15-μm tip (New Objective, Woburn, MA, USA). For database searching, MS raw data files were analyzed by Proteome Discoverer Software (version 1.4.1.14; Thermo Fisher, San Jose, CA, USA), and searched against the *T. vaginalis* ATCC PRA-98 reference genome (GenBank: AAHC00000000.2) using Mascot search engine (Matrix Science, London, UK; version 2.5).

The proteomics data obtained from all the *T. vaginalis* isolates were screened to identify core exosomal proteins. In addition, the Gene Ontology (GO) enrichment analysis was conducted to cluster all the identified proteins based on biological process, molecular function, or cellular component.

Results

Identification of exosomes released by six isolates of *Trichomonas vaginalis*

In this study, we have isolated exosomes from six isolates of *T. vaginalis*, ATCC 30236, 30238, 50148, 50143, PRA-98, and T1. These six *T. vaginalis* isolates can be considered as replicates in our study.

Fig. 1 shows the exosomes released by six *T.vaginalis* isolates under TEM after negative staining. TEM confirmed the presence of exosomes in all the *T. vaginalis* preparations. The observed exosomes isolated from different isolates showed a cup shape with bilayer membranes with the mean ranging from 100 to 150 nm in diameter. The isolated exosome size is similar to that reported by our previous studies.[24] Supplementary Fig. S1 supported the exosome sizes, which showed the average sizes of approximately 100 nm by Nanoparticle Tracking Analysis (NTA), using NanoSight NS300 (Malvern Instruments, Malvern, UK).

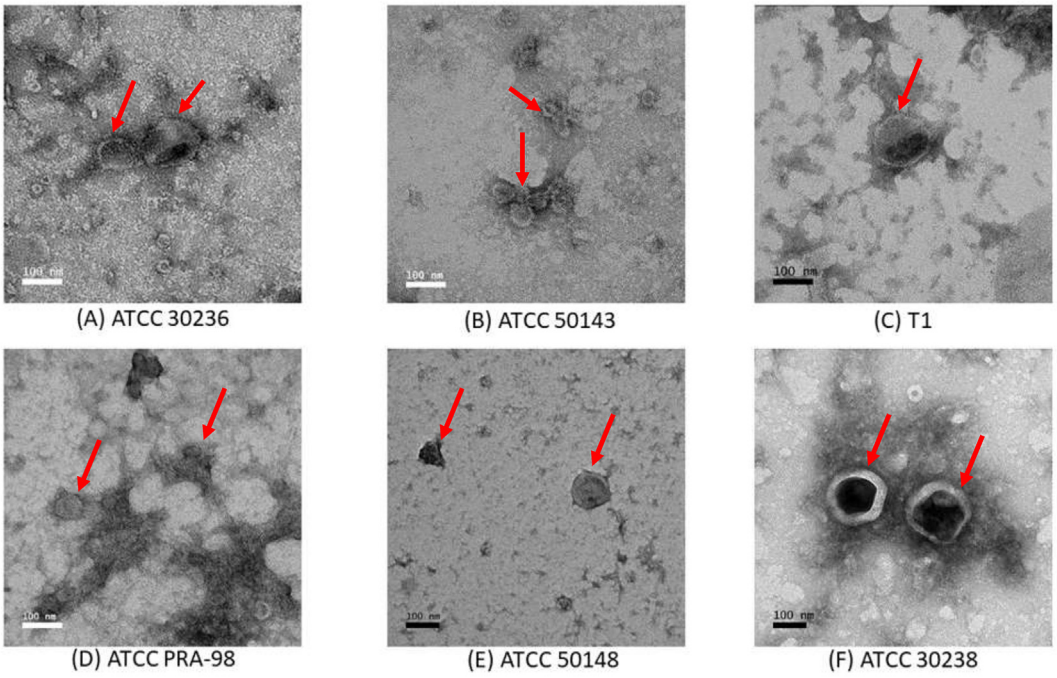

Figure 1. TEM images of the exosomes derived from six *T. vaginalis* isolates. The six isolates were (A) ATCC 30236, (B) ATCC 50143, (C) T1, (D) ATCC PRA-98, (E) ATCC 50148, and (F) ATCC 30238. (Scale bar = 100 nm). Arrows indicates the exosomes being observed.

Proteomic analysis in the exosomal extracts and supernatant fractions of six *Trichomonas vaginalis* isolates

To elucidate the exact core exosome proteome of *T. vaginalis*, we used the supernatant fraction of the same preparation as a control to eliminate 'non-core exosomal proteins'. We identified 1870 proteins from the enriched exosome extracts and the supernatant fractions of six isolates using LC-MS/MS (Fig. 2). Of the identified proteins, 227 (12%) were found only in the supernatant

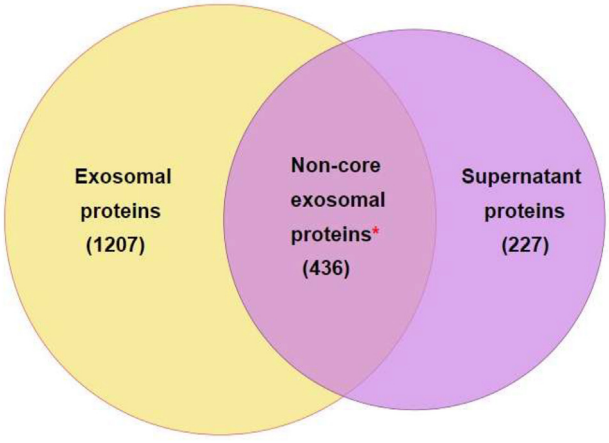

Figure 2. Venn diagram of total proteins identified from the exosomal extract by LC-MS/MS. The total identified proteins comprised the exosomal-specific proteins (1207 proteins), proteins from the supernatant fraction (227 proteins), and the proteins identified in both fractions (436 proteins).
Note: *Proteins not belonging to the core of the exosome.

Table 1 Common 'non-core exosomal proteins' found in the exosomal extracts.

Accession no.	Protein Name
TVAG_038420	conserved hypothetical protein
TVAG_043500	enolase, putative
TVAG_073860	phosphoenolpyruvate-protein phosphotransferase, putative
TVAG_113710	phosphoglycerate mutase, putative
TVAG_139300	phosphoenolpyruvate carboxykinase, putative
TVAG_190450	kakapo, putative
TVAG_212500	conserved hypothetical protein
TVAG_239310	bollus pemphigoid antigen, putative
TVAG_245580	conserved hypothetical protein
TVAG_253650	malate dehydrogenase, putative
TVAG_276410	translation elongation factor, putative
TVAG_293660	conserved hypothetical protein
TVAG_310250	phosphoenolpyruvate carboxykinase, putative
TVAG_347410	glyceraldehyde 3-phosphate dehydrogenase, putative
TVAG_348330	glycogen phosphorylase, putative
TVAG_393400	conserved hypothetical protein
TVAG_425470	conserved hypothetical protein
TVAG_573910	conserved hypothetical protein

Table 2 Seventy-two common exosomal-specific proteins found in the exosomal extracts from all of the six isolates.

Accession no.	Protein Name
TVAG_005910	50S ribosomal protein L2, putative
TVAG_006260	GTP-binding protein ypt10, putative
TVAG_008770	ubiquitin-protein ligase, putative
TVAG_009840	60S ribosomal protein L7a, putative
TVAG_015800	60S ribosomal protein L23, putative
TVAG_019180	TvTSP1
TVAG_020530	60S ribosomal protein L36e, putative
TVAG_021440	histone H2a, putative
TVAG_028010	dynamin, putative
TVAG_070500	Rab7g protein, putative
TVAG_075320	vacuolar proton ATPase, putative
TVAG_076170	26S proteasome regulatory subunit S3, putative
TVAG_076510	2-amino-3-ketobutyrate coenzyme A ligase, putative
TVAG_093600	60S ribosomal protein L23a, putative
TVAG_110530	40S ribosomal protein S20, putative
TVAG_121100	60S ribosomal protein L18, putative
TVAG_123140	interferon-induced GTP-binding protein mx, putative
TVAG_123500	STE family protein kinase
TVAG_149320	conserved hypothetical protein
TVAG_151500	chmp1, putative
TVAG_166580	conserved hypothetical protein
TVAG_169060	GTPase_rho, putative
TVAG_181000	RAB 19, 41 and, putative
TVAG_185160	60S ribosomal protein L19, putative
TVAG_197690	GTPase_rho, putative
TVAG_198960	60S ribosomal protein L9, putative
TVAG_209190	DEAD box ATP-dependent RNA helicase, putative
TVAG_211200	Rabx21 protein, putative
TVAG_213210	conserved hypothetical protein
TVAG_216890	vacuolar sorting protein, putative
TVAG_222450	Rabx18 protein, putative
TVAG_222520	ADP-ribosylation factor, arf, putative
TVAG_228640	histone H2b, putative
TVAG_240050	40S ribosomal protein sa, putative
TVAG_247230	hypothetical protein
TVAG_249850	RNAse L inhibitor, putative
TVAG_256510	Clan CA, family C2, calpain-like cysteine peptidase
TVAG_257310	Rab21, putative
TVAG_272960	40S ribosomal protein S24, putative
TVAG_282070	Rab8, putative
TVAG_282960	40S ribosomal protein S6, putative
TVAG_296770	tryptophanyl-tRNA synthetase, putative
TVAG_298300	dynamin, putative
TVAG_301380	GTP-binding protein alpha subunit, gna, putative
TVAG_305730	chaperone protein DNAj, putative
TVAG_311540	conserved hypothetical protein
TVAG_315190	CAMK family protein kinase
TVAG_328600	dock-10, putative
TVAG_329880	conserved hypothetical protein
TVAG_337240	actin, putative
TVAG_343970	50S ribosomal protein L5p, putative
TVAG_345870	conserved hypothetical protein
TVAG_347250	60S ribosomal protein L18a, putative
TVAG_350040	dynamin, putative
TVAG_351500	Rab7, putative
TVAG_367420	conserved hypothetical protein
TVAG_369310	conserved hypothetical protein
TVAG_388650	serine palmitoyltransferase I, putative
TVAG_389550	GTP-binding protein (q) alpha-11 subunit, gna11, putative
TVAG_394060	conserved hypothetical protein
TVAG_396200	neuroendocrine differentiation factor, putative
TVAG_414560	DTDP-glucose 4,6-dehydratase, putative
TVAG_421580	clathrin coat associated protein ap-50, putative
TVAG_445420	serine/threonine protein phosphatase, putative
TVAG_452120	GTP-binding protein alpha subunit, gna, putative
TVAG_454230	Rab15, 13, 10, 1, 35, 5, and, putative
TVAG_459530	vacuolar sorting protein SNF7, putative
TVAG_462480	rho4, putative
TVAG_477530	26S protease regulatory subunit S10b, putative
TVAG_478270	conserved hypothetical protein
TVAG_482290	small GTPase rabh, putative
TVAG_527180	RAB, putative

fractions. About 436 (23%) of the identified proteins exist both in the exosome and the supernatant fractions, and these proteins were termed as 'non-core exosomal proteins'. Table 1 shows 18 conserved 'non-core exosomal proteins' that were found in the exosomal extracts of all six isolates. These common 'non-core exosomal proteins' were mainly composed of conserved hypothetical proteins and proteins involved in carbohydrate, glucose, or pyruvate metabolic processes based on GO classification (Supplementary Fig. S2). All the details of the identified proteins, including accession number, protein name, and GO classification, were listed in Supplementary Table S1.

A total of 1207 (65%) exosomal-specific proteins were identified after eliminating the 'non-core exosomal proteins'. The functions of all these proteins were clustered by GO enrichment analysis (Supplementary Fig. S3). However, only 72 proteins could be identified in all six *T. vaginalis* exosome proteomes (Table 2). Therefore, we termed these proteins as 'common exosomal-specific proteins' in our study. According to the GO classification, the primary biological processes implicated included intercellular transport, establishment of cell localization,

and cellular localization. Meanwhile, cellular components like ribosome and ribonucleoprotein were observed. The majority of these common proteins played roles in molecular functions such as guanyl nucleotide binding, GTPase activity and GTP binding, which in fact could be categorized under the molecular function of catalytic activity (Fig. 3). Within the pool of 72 common exosomal-specific proteins identified in our findings, 8.3% were found to contain predicted transmembrane (TM) domains, while 1.4% were predicted to possess signal peptides (SP) (refer to Supplementary Table S2). Notably, this outcome closely aligns with the observations in Twu et al.'s study, where 9% were predicted to have TM domains and 3.7% were predicted to have SP.

In addition, Supplementary Table S3 showed the top 10 most abundant proteins in the six isolates' exosomal extracts. These proteins had the highest area of mass spectrometry peaks, with the peak area gained starting from approximately 10^9 to 10^8. Actin (TVAG_337240) was identified in all six isolates at the four highest areas (ranging from 2.5×10^8 to 4.2×10^9). The four most abundant proteins identified in isolates ATCC 30238 and ATCC 50148 were the actin cytoskeleton protein (TVAG_249200, TVAG_090470, TVAG_337240, and TVAG_054030). Elongation factor 1-alpha (TVAG_067400), which functions in translation and GTPase activity, was present in all the isolates except isolate T1.

Moreover, five proteins were not identified in ATCC 50143 but in all other isolates (Supplementary Table S4). There were mitochondrial genome maintenance 1 (MGM1) protein, a ribosomal protein, NAD dependent epimerase/dehydratase, DEAD box ATP-dependent RNA helicase and a conserved hypothetical protein.

Comparison of our results with three different proteomic studies

We also compared our proteomics study data with three other studies on sEV in *T. vaginalis*. Details of these experimental results, including total proteins, predicted transmembrane (TM) domains and signal peptides (SP), predicted common contaminants, and functional groups from each study can be found in Table 3.

In comparison with Twu et al.,[8] they identified 215 proteins in the exosome extract of the isolate B7RC2 using MudPIT-based proteomic mass spectrometry (MS). Whereas Govender et al.[25] identified 171 exosomal proteins and 21 contaminant proteins from sEV preparation of three TVV-positive isolates (347V+, UR1, and OC8) and three TVV-negative isolates (347V-, B7RC2 and OC7) by TMT-labeling MS. Besides, Rada et al.[22] identified 1633 proteins in the sEV preparation in TVV-positive clone TV79-49c1+ and isogenic TVV-free clone TV79-49c1-by Label-free quantitative mass spectrometry (LFQ-MS).

Fig. 4 summarised exosomal proteins identified from three published works[8,22,25] and this study in a Venn diagram. Only 16 core proteins were found to exist in two studies,[8,25] and our analysis. These core exosomal proteins included TvTSP1, actin, vacuolar sorting protein, ribosomal proteins and Rab GTPase proteins. The accession number and protein name of these proteins were listed in Table 4.

Discussion

We established the first large-scale comparative study on *T. vaginalis* exosome proteome from six isolates. In our study,

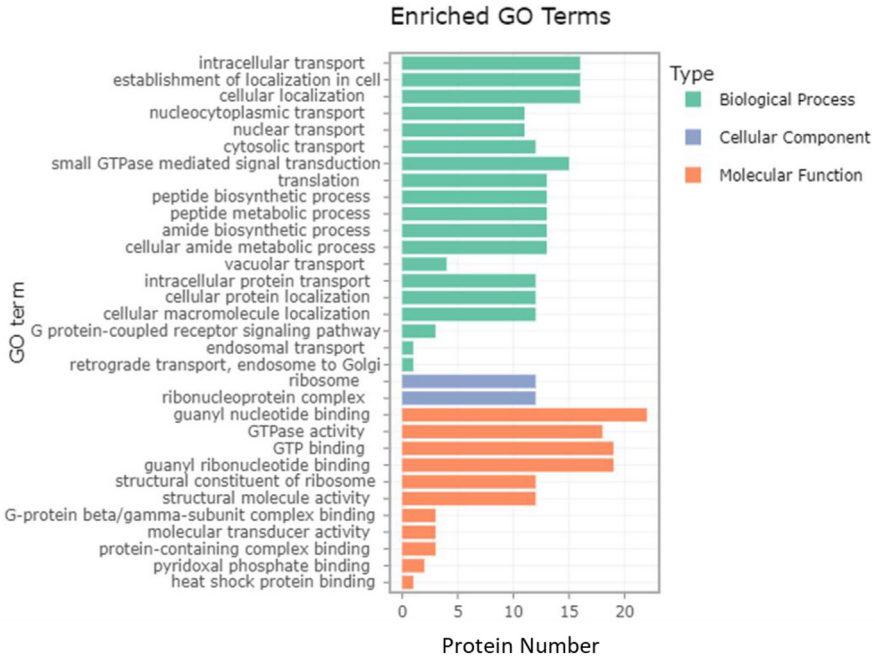

Figure 3. Summary of GO classification from the 72 common exosomal-specific proteins. Identified proteins were functionally categorized as biological process (green), cellular component (blue), and molecular function (orange color) based on GO classification.

Table 3 Comparison of our results with three different proteomic studies.

	Our study	Twu et al. (2013)	Govender et al. (2020)	Rada et al. (2022)
TV isolate	ATCC 30236, 30238, 50148, 50143, PRA-98, and T1	B7RC2	347V+, 347V-, UR1 clone, OC7, OC8, and B7RC2	TV79-49c1^{+} and TV79-49c1^{-} clone
Exosome isolation method	30% sucrose	Sucrose gradient	Gel filtration	Isotonic linear OptiPrep gradient
Proteomic analysis method	LC-MS/MS	MudPIT-based MS	TMT-labeling MS	Label-free quantitative-MS
Total protein identified	Total of 1207 proteins, with 72 consistently present in the exosome preparations from all 6 TV isolates	215 proteins	Total of 241 proteins were identified with 171 proteins consistently present in the sEV preparations from all 6 TV isolates (after excluded 21 contaminant proteins)	1633 proteins
Control group	Supernatant proteins	N.A.	N.A.	N.A.
Common contaminants	23% (436 proteins; experimental)[a]	N.A.	11% (21 proteins; predicted)	N.A.
TM and SP predicted	TrichDB:- 8.3% (6/72) TM; 1.4% (1/72) SP	TrichDB: 9% (19/215) TM; 3.7% (8/215) SP	N.A.	N.A.
Functional groups based on GO	GO:- The 3 largest molecular function involved guanyl nucleotide binding (22/72), GTP binding (19/72) and guanyl ribonucleotide binding (19/72). The 3 largest biological process involved intercellular transport, establishment of localization in cell and cellular localization (16/72 each).	GO:- 14% were signaling proteins, 14% were metabolic enzymes; 13% were cytoskeletal proteins; 8% involved in transport; 6% were vacuolar proteins; 15% were hypothetical proteins; and 24.6% were *T. vaginalis* surface proteome.	GO:- By molecular function, 24.0% involved in catalytic activity and 21.1% involved in binding. By protein class, 17 classess were assigned with the larger proportions belonging to protein binding and modifying, metabolite enzyme, extracellular matrix and defense/imunity.	COGs:- 18 functional categories: The largest (17%) involved in intracellular trafficking; 14% were peptidases; 9% were involved in signal transduction mechanism.

[a] Non-core exosomal proteins (not belonging to the core of the exosome).
Abbreviations: TM = Transmembrane domain protein; SP= Signal peptide; TV = *T. vaginalis*; N.A. = Non-available; GO= Gene Ontology; COGs = Clusters of orthologous groups.

a total of 1870 exosomal proteins were identified by LC/MS mass spectrometry. However, only 1207 proteins were confirmed exosomal-specific after excluding 'non-core exosomal proteins' from the supernatant fraction. Among these 1207 exosomal-specific proteins, 72 were identified in the exosomal extracts from all six *T. vaginalis* isolates and termed as 'common exosomal-specific proteins'.

How to remove 'non-core exosomal proteins' in exosomal extracts is a major hurdle in exosome proteomics research. Most studies on *T. vaginalis* exosome proteome rely on previously reported contaminants as a reference. This study uses the supernatant fraction as a control to identify exosomal-specific proteins. There were 436 (23%) 'non-core exosomal proteins' found in both exosome and supernatant fractions determined in our study.

The recent study identified 72 common exosomal-specific proteins from six *T. vaginalis* isolates. Whereas only 16 core exosomal-specific proteins were found when comparing our study with three published studies.[8,22,25] We were incapable to compare a complete dataset from other studies with our proteome, while our dataset shared only 55 proteins with that of Rada et al.[22] Various factors such as different culture conditions, diverse environmental conditions or exosome isolation methods, sample processing and database screened could contribute to the variability in sEV composition and proteomic analyses of exosome. Subsequently, we used 30% sucrose based on Gupta et al.[27] study to isolate exosome instead of sucrose or OptiPrep gradient from other studies.[8,22] In our study, we followed cultivation methods based on Twu et al.[8.] The isolates were cultivated

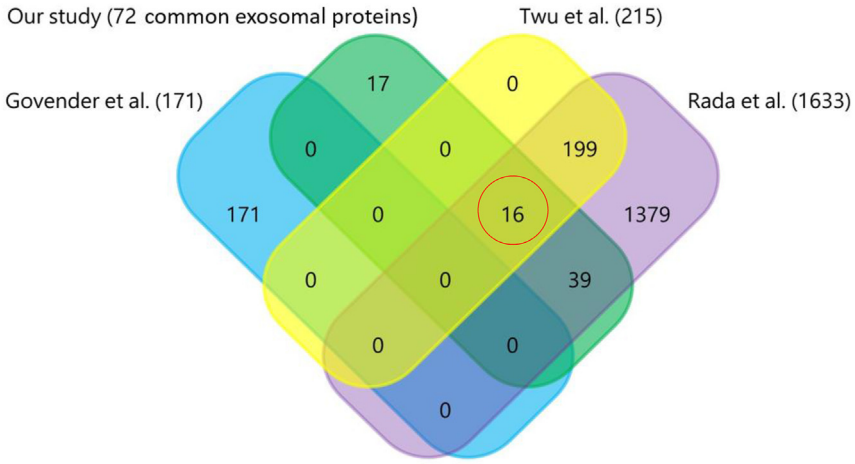

Figure 4. Venn diagram of core exosomal proteins identified from four studies. Rada et al. identified a total of 1633 proteins (purple), Twu et al. identified a total of 215 proteins (yellow), Govender et al. identified a total of 171 proteins (blue), compared with 72 common exosomal-specific proteins from our study (green). Among these, 16 core proteins were characterized.

Table 4 List of 16 core proteins which found from comparison with other three studies.

Accession no.	Protein Name
TVAG_019180	TvTSP1
TVAG_070500	Rab7g protein, putative
TVAG_151500	chmp1, putative
TVAG_169060	GTPase_rho, putative
TVAG_197690	GTPase_rho, putative
TVAG_222450	Rabx18 protein, putative
TVAG_282070	Rab8, putative
TVAG_301380	GTP-binding protein alpha subunit, gna, putative
TVAG_337240	actin, putative
TVAG_347250	60S ribosomal protein L18a, putative
TVAG_351500	Rab7, putative
TVAG_367420	conserved hypothetical protein
TVAG_389550	GTP-binding protein (q) alpha-11 subunit, gna11, putative
TVAG_396200	neuroendocrine differentiation factor, putative
TVAG_452120	GTP-binding protein alpha subunit, gna, putative
TVAG_459530	vacuolar sorting protein SNF7, putative

until a certain cell density of 2×10^6 cells/mL and transferred into serum-free YIS medium, and subsequently were harvested after 4 h incubation for exosome isolation. In addition, our cultivation temperature was set at 37 °C continuously. The identification of proteome in *T. vaginalis* is similar with those three compared studies,[8,22,25] where the proteins analysed by LC/MS were sorted based on TrichDB annotations and Pfam domain identification. Although the culture conditions were the same for the six isolates, there was a limitation that the exosomes originating from an axenic culture will not be similar to exosomes originating from in vivo infections. Therefore, the in vivo exosomal protein composition is likely to be different due to variable growth conditions of the parasite.

The 16 core exosomal-specific proteins identified here were tetraspanin TvTSP1, ribosomal proteins, Rabs, subunits of heterotrimeric G proteins, which were pathogenesis related proteins involved as virulence factor cargo. Most of these core exosomal-specific proteins were also classical markers of exosomes. In addition, other core exosomal-specific proteins such as actin, thioredoxin peroxidase, metalloprotease, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and glutathione S-transferase have also been identified in the context of parasite-derived EVs.[30,31]

Tetraspanin (TSP) is the classical marker for exosomes. TvTSP1 (TVAG_019180) was present in our exosome preparations from all six isolates. TSPs are cell surface proteins participating in adhesion, cell proliferation, signal transduction, migration, and host–parasite interactions.[32] TvTSP1 was upregulated in EVs upon parasite exposure to vaginal epithelial cells, suggesting that TvTSP1 may be involved in the host–parasite interaction.[8,15] Moreover, TvTSP1 also resides firmly on the surface (plasma membrane and flagella) and internally in the membranes of intracellular vesicles.[8,33]

Another surface protein vATPase (TVAG_075320) previously reported by de Miguel et al.[20] and Salas et al.[11] was also identified in Rada et al.[22] and our study. However, our study did not detect the novel surface proteins of BspA family (TVAG_240680) and metallopeptidase (TVAG_224980), which may be involved in pathogenesis.[8] Another protein, ESCRT-III subunit vacuolar protein sorting-associated protein 32 (VPS32; TVAG_459530) known as SNF7[8,15,22] and a component of the ESCRT machinery, was identified in our study as well as two other studies.[8,22] Notably, Salas et al.[11] revealed that VPS32 plays a crucial role in regulating parasite attachment to prostate cells contributing to *T. vaginalis* pathogenesis, and is important in biogenesis for EV formation.

Orthologs of virulence proteins characterized in other parasites (TVAG_371800, peptidase M8, leishmanolysin or GP63-like) which observed in exosome proteome in Twu et al.[8] study was also found in our six isolates of *T.*

vaginalis. The potentially protein involved in host immune regulation (TVAG_137880, peptidyl proyl isomerase A or cyclophilin A) which was notable in Twu et al.[8] study, was also identified in our exosomal samples of five isolates except ATCC PRA-98.

The four most abundant proteins identified in isolates ATCC 30238 and ATCC 50148 were TVAG_249200, TVAG_090470, TVAG_337240 and TVAG_054030, which were grouped under actin family. TVAG_337240 (actin) was identified in all the six isolates in this study. Actin is ATPase and nucleotide-binding domain that mediates flagellate-amoeboid transition and migration across host tissue upon inflection.[34] In addition, actin is also associated with cytoskeletal microfilaments.[35] Therefore, the presence of structural proteins in exosomes proposed that they may be related to the production of vesicles.

Ribosomal proteins are multifunctional proteins that may function in structural molecular activity, transcription, translation, and protein synthesis. More than 80 ribosomal proteins were predicted in the *T. vaginalis* genome.[36] Fourteen (19.4%) of the 72 common exosomal-specific proteins were the 40S, 50S, and 60S ribosomal proteins (Supplementary Table S3). TVAG_067400 (60S ribosomal protein), was present highly in all the isolates except isolate T1. In comparison, TVAG_347250 (60S ribosomal protein L18a) was found in our study and the other two studies.[8,22]

Some proteins, such as members of the Rab GTPase family (Rab 1, 5, 7, 8, 10, 13, 15, 19, 41) involved in vesicle biogenesis, are also present in our exosome proteome. Rab proteins are the largest family of small GTPases, and have been identified in previous studies in helminths[37] and other organisms.[38] They regulate intracellular vesicle transport through processes like budding, mobility along the cytoskeleton, and membrane fusion. Different Rab proteins are involved in various stages of exosome secretion pathway. For example, Rab4, Rab5, and Rab11 are involved in the early stages, while Rab7 and Rab9 are in the later stages.[39] Additionally, the exosomal protein TVAG_452120 (GTP-binding protein alpha subunit) found to localize in large vesicular structures in *T. vaginalis*,[40] has been identified in various exosomal studies, including Twu et al.,[8] Rada et al.[22] and our study.

Based on GO classification when sorting into molecular function groups, most identified proteins in our study were involved in catalytic activity and followed by binding function, which was similar to the study of Govender et al.[25]

On the contrary, two proteins, Mgm1 (Mx proteins) and the DEAD-box RNA Helicase DDX3, were notably absent in ATCC 50143 (a virus-negative isolate) but present in other TVV-positive isolates. Mgm1 proteins (TVAG_047650) function as dynamin-like large GTPases, playing a crucial role in antiviral activity according to Verhelst et al.,[41] and mediating mitochondrial inner membrane fusion as demonstrated by Abutbul-Ionita et al.[42] Notably, Mgm1 was found to be less abundant in sEV samples isolated from virus-negative isolates (347V-, OC7, and B7RC2) in the study by Govender et al.[25] and it was associated with catalytic activity, metabolic processes, and neutrophil degranulation. However, the presence of Mgm1 was not significantly different in both TVV-positive (TV79-49c1+) and negative (TV79-49c1-) clones in the study by Rada et al.[22]

On the other hand, DEAD-box helicases (TVAG_380910) constitute a large family of conserved RNA-binding proteins belonging to the broader group of cellular DExD/H helicases. These helicases are present in protozoa and helminth parasites, such as *P. falciparum, L. major, Trypanosoma* spp., *G. lamblia, Entamoeba histolytica, and B. malayi*.[43] DEAD-box helicases play critical roles in transcription, cellular RNA metabolism, and translation. Additionally, they act as components of antiviral innate immunity by binding with viral RNA[44] and can manipulate viruses to facilitate their replication.[45]

Conclusions

Exploring exosomes and their proteome represents a rapidly expanding field in research, holding significant potential applications in medicine and biology. While some evidence suggests a potential role of exosomes in the pathogenesis of *T. vaginalis* infection, a more comprehensive understanding of the contents of *T. vaginalis* exosomes is crucial to elucidate the roles of exosomes in disease and explore their potential as diagnostic or therapeutic targets. This study employed LC-MS/MS to investigate the protein composition of enriched exosomal extracts and supernatant fractions from six *T. vaginalis* isolates. The result is the generation of the most extensive *T. vaginalis* exosome proteome dataset to date. Notably, the study highlighted the importance of using the supernatant fraction as a control to eliminate 'non-core exosomal proteins' in the exosome proteome.

The research identified 72 common exosomal-specific proteins consistently present in exosomes. Among these proteins were tetraspanins, membrane proteins integral to exosome formation and crucial in recognizing exosomes by target cells. Additionally, various proteins with catalytic activity and binding functions were identified. However, aligning our experimental findings with similar studies proved challenging. The variability in exosome proteomes can be attributed to several factors, particularly the diverse exosome isolation and enrichment methods. Furthermore, many previous studies lacked adequate experimental controls to eliminate 'non-core exosomal proteins' from the supernatant fraction after ultracentrifugation. The experimental design of our study emphasizes the critical role of utilizing the supernatant fraction as a control in exosome proteomic investigations. This approach enhances the reliability and specificity of exosomal protein identifications, contributing to a more accurate understanding of the complex landscape of *T. vaginalis* exosomes and their potential implications for health and disease.

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Declaration of competing interest

All authors declare that they have no competing interests.

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Appendix A. Supplementary data

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