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The generation gap: Proteome changes and strain variation during encystation in *Giardia duodenalis*

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ABSTRACT

The prevalence of *Giardia duodenalis* in humans is partly owed to its direct and simple life cycle, as well as the formation of the environmentally resistant and infective cysts. Proteomic and transcriptomic studies have previously analysed the encystation process using the well-characterised laboratory genomic strain, WB C6. This study presents the first quantitative study of encystation using pathogenically relevant and alternative assemblage A strains: the human-derived BRIS/82/HEPU/106 (H-106)and avian-derived BRIS/95/HEPU/2041 (B-2041). We utilised tandem MS/MS with a label-free quantitative approach to compare cysts and trophozoite life stages for strain variation, as well as confirm universal encystation markers of assemblage A. A total of 1061 non-redundant proteins were identified from both strains, including trophozoite- and cyst-specific proteomes and life-stage differentially expressed proteins. Additionally, 24 proteins previously classified in the literature as encystation-specific were confirmed as strain-independent markers of encystation. Functional cluster analysis of differentially expressed proteins saw significant overlap between strains, including protein trafficking and localisation in cysts, NEK kinase function, and carbohydrate metabolism in trophozoites. Two significant points of strain specific adaptations in cysts were also identified. B-2041 possessed major up-regulation of the ankyrin repeat protein 21.1 family compared to H-106. Furthermore, cysts of B-2041 retained near-complete VSP variant diversity between cysts and trophozoites, while H-106 lost 45% of its VSP variant diversity between life cycle stages, a constriction previously observed in studies of WB C6. This is the first report of strain variation in the cyst stage in *G. duodenalis*, and highlights cyst variation and its impacts on reinfection and life cycle success.

1. Introduction

Giardia duodenalis is a flagellated protozoan responsible for a global pandemic afflicting 250 million of the world's population at any one time, making it the most common diarrheal parasite. *G. duodenalis* is the perfectly adapted parasite associated with short-term morbidity rather than mortality, as it is adapted to a wide range of host species and displays zoonoses overlapping those of other closely related *Giardia* species [1]. At the heart of the evolutionary success of *G. duodenalis* is the process of cyst formation. *G. duodenalis* has a simple, direct life cycle comprising discrete parasitic trophozoites and cyst stages with transmission predominantly mediated by contaminated waterborne routes. The cyst is the environmentally resistant infective form (ERIF), formed in the jejunum and shed in the faeces, making it essential for life cycle completion. Cysts are able to accumulate and persist in the environment for months at a time awaiting ingestion, even resisting some disinfectants, until primed by host gastric signals which prompt trophozoite emergence [2].

Encystation is a complex process involving significant amounts of cell structure remodelling and metabolic adjustment. Tear-dropped trophozoites, which are flagellated and binucleate (4N), become increasingly rounded, lose the ability to attach to host cells,

Abbreviations: ANK, ankyrin; CW, cyst wall; CWP, cyst wall protein; ES, enrichment score; ESV, encystation-specific vesicle; FDR, false discovery rate; GalNAc, β(1-3)-*N*-acetyl-D-galactosamine; GO, gene ontology; GPF, gas phase fractionation; HCMP, high cysteine membrane protein; Nano LC–MS/MS, nanoflow liquid chromatography tandem mass spectrometry; NSAF, normalised spectral abundance factor; ORF, open reading frame; PDI, protein disulfide isomerase; PDX, peroxiredoxin; P.I, post induction; PV, peripheral vesicle; SAGE, serial analysis of gene expression; SpC, spectral count; TCP-1, chaperonin T complex 1; TMT, tandem mass tag; VSP, variable surface protein.

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internalise their flagella, disassemble median bodies and become immotile cysts with 4 nuclei and 16N ploidy [3,4]. Trophozoites bound for encystation are shunted out of the cell-cycle at the G_2-M transmission to begin the process, which takes approximately 20–24 h [5]. Early encystation is characterised by cyst wall (CW) formation and the presence of encystation-specific vesicles (ESV) within the first 8–10 h post induction (p.i). The CW is comprised of three cyst wall proteins (CWP), CWP1-3, which are synthesised, concentrated and transported in the ESV to be complexed with β(1-3)-*N*-acetyl-D-galactosamine (GalNAc) polymer (~60% of the CW) to produce the 300 nm thick CW [4,6]. Encystation is triggered *in vitro* by simulating conditions of the mid-lower portion of the jejunum. *In vitro* encystations predominately utilise high bile concentrations and high pH, or cholesterol starvation, but may also involve addition of lactic acid, primary bile salts and deprivation of bile prior to induction [5,8,9]. Currently, the specific factors that induce encystation in bile, an unfractionated non-standardised media addition, remain unknown. Additionally, *in vitro* encystation is not universally induced under such conditions, as some laboratory passaged strains and clones are non-responsive to *in vitro* signals and fail to encyst [7]. The induction of encystation in the host, where host immune pressures may contribute to, or influence, the cyst formation process, is yet uncharacterised.

To date, our understanding of regulation of *Giardia* encystation through proteomic and transcriptomic studies is limited to the *G. duodenalis* laboratory strain WB strain first isolated in 1979, or the laboratory optimal and clonally derived WB C6 (ATCC 50803) generated in 1983. So far, microarray analyses of cysts and trophozoites have revealed a small set of 18 encystation genes up-regulated independent of encystation method used [5], while further microarray analysis revealed a limited transcriptome within cysts, implying metabolic dormancy [8]. Recently, Serial Analysis of Gene Expression (SAGE) was used to monitor changes in mRNA abundance across multiple timepoints during both encystation and excystation, allowing a global snapshot of the changes throughout key life cycle processes [9]. Using mass-spectrometry based quantitative proteomics, cysts and trophozoites have been previously compared using 2DE gel electrophoresis [10], as well LC–MS/MS proteomic analysis of encystations across 14 h using label-free quantitation [6]. The increasing number of post-genomic technologies quantitating gene and protein expression are amalgamating a reproducible core set of stage-specific markers throughout the multiple stages of encystation, but only using WB C6 as the best characterised laboratory strain [6]. While this provides pivotal information it fails to provide an adequate understanding of genomic, transcriptomic and proteomic transition in alternative and pathogenically relevant strains. Furthermore, with wide variations in host range and zoonoses, as well as experimental evidence of varying cyst infectivity [11], it is highly likely that strain variations during encystation occur to impact life cycle completion and zoonotic potential in subsequent generations.

The present study reports the first comparative and quantitative proteomic analysis of cysts and trophozoites from two alternate assemblage A strains, specifically derived from pathogenic strains of diverse host specificity. We have utilised two Australian *G. duodenalis* strains from the A1 sub-assemblage, BRIS/82/HEPU/106 (H-106), isolated from a diarrhoeic child, and BRIS/95/HEPU/2041 (B-2041), which was isolated from a wild-caught cockatoo (*Cacatua galerita*) who succumbed to terminal enteritis [12]. Both these strains have well characterised phenotypes and infection models in the literature, with B-2041 showing a more intense pathogenicity and pathophysiology compared to H-106 [13–15]. This makes this study the first to consider encystation processes outside the genomic strain WB C6, in the search for reproducible and pathogenically relevant markers of the *Giardia* life cycle.

2. Methods

2.1. Parasite culture and encystation

Axenic cultures of trophozoites of H-106 and B-2041 were grown in TYI-S-33 medium supplemented with 1% bile and newborn calf serum [16] as previously described [17]. Parasites were subcultured at end-log phase into fresh media, and grown and harvested for protein extraction within 5 passages of recovery from cryopreservation. Three biological replicates generated from separate cultures were grown for each strain and encysted and harvested separately. Absence of bacterial and fungal contamination was verified using serial dilutions and streak plates to ensure no colony forming units were detected in cultures prior to encystation and extraction.

Half of each triplicate culture was harvested to extract the trophozoite proteins, and the remaining half progressed through to encystation. *Giardia* trophozoites were harvested from pre-confluent cultures in by chilling on ice with vortexing for 15 min to detach parasites from the walls of the culture vessel. Trophozoites were harvested by centrifugation at 3000 × *g* for 10 min, then washed twice more with ice-cold PBS to remove media traces [14]. Encystation conditions were optimised to maximise cyst yield as previously described for each strain [7,18]. Maximum Type 1 cysts for H-106 were achieved using a 24 h/24 h encystation to growth media method [7], and 40 h in encystation media was used for the slower growing B-2041 [18]. Both strains were incubated in encystation media containing high bile, lactic acid and increased pH of 7.8 [18]. After encystation, cysts were enriched using hypotonic lysis, washed twice in dH_2O to remove cell debris and collected using sedimentation and centrifugation.

2.2. Protein extraction, digestion and peptide extraction

Trophozoites and cysts were extracted in ice-cold SDS sample buffer containing 1 mM EDTA and 5% beta-mercaptoethanol, and then reduced at 75 °C for 10 min. Protein extracts were centrifuged at 0 °C at 13,000 × *g* for 10 min to remove debris, and stored at −20 °C. The concentration of protein in each solution was measured by BCA assay (Pierce) before fractionation and digestion. For each sample 250 μg of protein was extracted from SDS sample buffer using chloroform-methanol [19], with the protein pellet washed 2–3 times with methanol. In-solution digestion was performed using the filter aided sample preparation (FASP) method [20,21], modified to solubilise protein in 2,2,2-trifluoroethanol (TFE) [22] as previously described [23]. Each extract was reconstituted to 60 μL with 2% formic acid, 2% TFE and stored at −20 °C until analysis by LC–MS/MS.

2.3. Theoretically derived GPF calculations using predictive software

Optimised GPF mass ranges were calculated using theoretical trypsin digest according to Scherl et al. [24], with the Giardiadb.org 2.5 release of the A1 genome (Strain WB) with charge states +2 and +3 considered, as well as carbamidomethyl modifications. The mass ranges were calculated as following: the low mass range was 400–520 amu, the low-medium mass range was 515–690 amu, the medium-high mass range was 685–990 amu and the high mass range was 985–2000 amu.

2.4. Nanoflow LC–MS/MS

Each FASP protein digest was analysed as 4 repeat injections across the four mass ranges as calculated in section 2.3, on a LTQ Velos Pro linear ion trap mass spectrometer (Thermo, San Jose CA).