Journal of Proteomics xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Journal of Proteomics

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

Integrative transcriptome and proteome analyses define marked differences between *Neospora caninum* isolates throughout the tachyzoite lytic cycle

P. Horcajo[a,1], D. Xia[b,1], N. Randle[c], E. Collantes-Fernández[a], J. Wastling[d], L.M. Ortega-Mora[a], J. Regidor-Cerrillo[a,*]

[a] SALUVET, Animal Health Department, Faculty of Veterinary Sciences, Complutense University of Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain
[b] The Royal Veterinary College, Royal College Street, London NW1 0TU, United Kingdom
[c] Department of Infection Biology, Institute of Infection & Global Health, University of Liverpool, Liverpool Science Park IC2, 146 Brownlow Hill, Liverpool L3 5RF, United Kingdom
[d] Faculty of Natural Sciences, Keele University, Keele, Staffordshire ST5 5BG, United Kingdom

ARTICLE INFO

Keywords:
Neospora caninum
Low and high virulence isolates
Proteome
Transcriptome

ABSTRACT

Neospora caninum is one of the main causes of transmissible abortion in cattle. Intraspecific variations in virulence have been widely shown among *N. caninum* isolates. However, the molecular basis governing such variability have not been elucidated to date. In this study label free LC-MS/MS was used to investigate proteome differences between the high virulence isolate Nc-Spain7 and the low virulence isolate Nc-Spain1H throughout the tachyzoite lytic cycle. The results showed greater differences in the abundance of proteins at invasion and egress with 77 and 62 proteins, respectively. During parasite replication, only 19 proteins were differentially abundant between isolates. The microneme protein repertoire involved in parasite invasion and egress was more abundant in the Nc-Spain1H isolate, which displays a lower invasion rate. Rhoptry and dense granule proteins, proteins related to metabolism and stress responses also showed differential abundances between isolates. Comparative RNA-Seq analyses during tachyzoite egress were also performed, revealing an expression profile of genes associated with the bradyzoite stage in the low virulence Nc-Spain1H isolate. The differences in proteome and RNA expression profiles between these two isolates reveal interesting insights into likely mechanisms involved in specific phenotypic traits and virulence in *N. caninum*.

Significance: The molecular basis that governs biological variability in *N. caninum* and the pathogenesis of neosporosis has not been well-established yet. This is the first study in which high throughput technology of LC-MS/MS and RNA-Seq is used to investigate differences in the proteome and transcriptome between two well-characterized isolates. Both isolates displayed different proteomes throughout the lytic cycle and the transcriptomes also showed marked variations but were inconsistent with the proteome results. However, both datasets identified a pre-bradyzoite status of the low virulence isolate Nc-Spain1H. This study reveals interesting insights into likely mechanisms involved in virulence in *N. caninum* and shed light on a subset of proteins that are potentially involved in the pathogenesis of this parasite.

1. Introduction

Neospora caninum is a cyst-forming obligate intracellular protozoan parasite that is closely related to *Toxoplasma gondii*, which infects different domestic or wild canids as its definitive host and cattle and other ungulates as intermediate hosts [1]. *N. caninum* has been recognized as one of the main causes of abortion in cattle, resulting in devastating economic losses to the beef and dairy industries [2]. Although various factors are potentially involved in determining the dynamics of *N. caninum* infection, experiments in pregnant cattle have shown the key role of different isolates of *N. caninum* in the severity of disease and its capacity to cause foetal mortality in cattle [3–6]. Host tissue damage occurs as a consequence of the tachyzoite lytic cycle, a process that enables parasite propagation and involves the following successive steps: parasite invasion, adaptation to new intra-cytoplasmatic conditions, intracellular proliferation and egress from host cells [7,8]. Interestingly, the *in vitro* behaviour of a *N. caninum* population in these processes has demonstrated the potential association of the phenotypic

* Corresponding author at: Animal Health Department, Faculty of Veterinary Sciences, Complutense University of Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain.
E-mail addresses: phorcajo@ucm.es (P. Horcajo), dxia@rvc.ac.uk (D. Xia), Nadine.Randle@liverpool.ac.uk (N. Randle), esthercf@ucm.es (E. Collantes-Fernández), j.wastling@keele.ac.uk (J. Wastling), luis.ortega@ucm.es (L.M. Ortega-Mora), jregidor@ucm.es (J. Regidor-Cerrillo).
[1] Authors contributed equally.

https://doi.org/10.1016/j.jprot.2017.11.007
Received 9 August 2017; Received in revised form 25 October 2017; Accepted 9 November 2017

traits such as the invasion rate and tachyzoite yield with pathogenicity observed in animal models [9–11]. Nevertheless, the molecular basis and mechanisms that govern such biological diversity in *N. caninum* remain largely unknown. *N. caninum* appears to be highly conserved genetically [12], although previous proteomic approaches have identified some differences between isolates [13–15]. Differences in secretory elements (rhoptry and dense granule proteins) and protein related to gliding motility and oxidative stress have been described among *N. caninum* isolates showing variations in protein expression, post-translational modifications and protein turnover [15]. Recently, an *in vitro* study comparing host cell modulation by *N. caninum* isolates with high (Nc-Spain7) and low (Nc-Spain1H) virulence has shown a great similarity in host transcriptome modulation by both isolates but marked differences in the parasite transcriptome between isolates [16]. In this study, we used a global approach to examine the changes between the *N. caninum* Nc-Spain7 and Nc-Spain1H isolates throughout the fast replicating tachyzoite lytic cycle. We exploited label free LC-MS/MS technology to investigate in deep proteome differences across the tachyzoite lytic cycle: after tachyzoite invasion and adaptation in the host cell at 12 h post infection (hpi), during active parasite replication at 36 hpi and at early egress at 56 hpi. Furthermore, we analysed the transcriptome status of Nc-Spain7 and Nc-Spain1H using RNA-Seq during tachyzoite egress from the host cell. We determined specific patterns of protein abundance for each isolate in each phase of the lytic cycle studied and differences between gene expression profiles that reveal interesting insights into differences in virulence between these two isolates.

2. Materials and methods

2.1. Parasite culture

Parasites were cultured in confluent Marc-145 cultures as previously described [17]. Briefly, medium from Marc-145 cultures grown for 24 h in DMEM with 10% of heat inactivated FBS and 1% antibiotic-antimycotic solution (Gibco, Gaithersburg, MD, USA) was replaced with DMEM supplemented with 2% FCS and 1% of antibiotic-antimycotic solution. Then, cell monolayers were inoculated with an adjusted multiplicity of infection (MOI) of Nc-Spain1H and Nc-Spain7 tachyzoites for parasite passaging onto a new Marc-145 monolayer each three – four days. All experiments in this study were conducted with tachyzoites from both isolates with a limited number of passages (Nc-Spain1H and Nc-Spain7, passage 13–18). All inoculations in *in vitro* assays were performed within 1 h after tachyzoite collection from flasks.

2.2. Experimental design and tachyzoite production for proteome and transcriptome analyses

The overall experimental design is shown in Fig. 1. All experiments were carried out with three biological replicates.

Confluent 24-h Marc-145 DMEM free of phenol red (Gibco, Gaithersburg, MD, USA) and FBS were inoculated with purified Nc-Spain1H tachyzoites at a MOI of 7 and Nc-Spain7 tachyzoites at a MOI of 4. Cell monolayers were recovered at 12 hpi (after completion of invasion and prior to tachyzoite duplication), at 36 hpi (active proliferation in the parasitophorous vacuole) and at 56 hpi (early egress), from T75 cm^2 flasks by cell scraping in 5 ml of PBS supplemented with protease and phosphatase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA), passaged by 25 G needles for host cell disruption and purified using PD-10 (Sephadex G-25 columns -GE-Healthcare, Barrington, IL, USA). Tachyzoite purification was carried out at 4 °C. The number and viability of tachyzoites was determined by trypan blue exclusion followed by counting in a Neubauer chamber. Tachyzoites were pelleted by centrifugation at 1350 × *g* for 10 min and stored at − 80 °C until tachyzoite proteome (TZP) analysis.

Tachyzoite samples for transcriptome analysis were obtained as described above. Cell cultures were recovered at 56 hpi, and tachyzoites were purified using PD10 columns as described above. Tachyzoite pellets were directly resuspended in 300 µl of RNAlater (Invitrogen, Carlsbad, CA, USA) and stored at − 80 °C until RNA extraction.

The tachyzoite growth and lytic cycle was monitored daily by microscopy, and photomicrographs for each time-point of sample collection were obtained at 400 × on an inverted microscope (Nikon Eclipse E400) connected to a digital camera for checking lytic cycle progression and sample collection in the programmed lytic cycle phases (Fig.1A).

2.3. LC-MS/MS analyses

Detailed materials and methods for sample preparation, LC-MS/MS, proteome data analysis, and Western blot validation are shown in Supplementary File 1. Briefly, prior to trypsin digestion, tachyzoite pellets were resuspended in 25 mM ammonium bicarbonate and RapiGest™ (Waters MS Technologies, Milford, MA, USA) for protein solubilization, reduced with DTT and alkylated with iodocetamide for trypsin digestion. Then, the digests were analysed using an LC-MS/MS system comprising an Ultimate 3000 nano system coupled to a Q-Exactive mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Reversed-phase liquid chromatography was performed using the Ultimate 3000 nanosystem by a linear gradient of 5–40% solvent B (80% acetonitrile in 0.1% formic acid) in 0.1% formic acid (solvent A). The Q-Exactive was operated in data-dependent mode with survey scans acquired at a resolution of 70,000 at *m/z* 200. Up to the top 10 most abundant isotope patterns were selected and fragmented by higher energy collisional dissociation with normalized collision energies of 30. The maximum ion injection times for the survey scan and the MS/MS scans were 250 and 100 ms, respectively.

For proteome data analyses, the Thermo RAW files were imported into Progenesis QI (version 2.0, Nonlinear Dynamics, Durham, CA, USA). Replicate runs were time-aligned using default settings and an auto-selected run as a reference. Spectral data were transformed into .mgf files with Progenesis QI and exported for peptide identification using the Mascot (version 2.3, Matrix Science, London, UK) search engine and the database ToxoDB-26_N*caninum* LIV_Annotated Proteins (version 26, ToxoDB). The false discovery rates were set at 1% and at least two unique peptides were required for reporting protein identifications. Finally, protein abundance (iBAQ) was calculated as the sum of all the peak intensities (from the Progenesis output) divided by the number of theoretically observable tryptic peptides for a given protein (Fig. 1B).

The mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium *via* the PRIDE [18] partner repository with the dataset identifier PXD007062.

The identified proteins were classified according to their parasite localization and functionality according to the Gene Ontology (GO) terms (annotated and predicted) on the ToxoDB website [19] for the Nc-Liverpool isolate (ToxoDB-26_N*caninum*LIV_AnnotatedProteins), *T. gondii* syntenic homologues (version 26, ToxoDB) and previous reports [20–22].

Validation of LC-MS/MS results was performed by measuring the differential abundance of the proteins MIC2 (NCLIV_022970), ROP2 (NCLIV_001970), and NTPase (NCLIV_068400) between isolates by Western blot analyses using the SAG1 protein (NCLIV_033230) as a housekeeping gene as previously described [23,24]. Images from WB membranes were obtained using a GS-800 Scanner (Bio-Rad Laboratories, Hercules, CA, USA) and were analysed with Quantity One quantification software v. 4.0 (Bio-Rad Laboratories, Hercules, CA, USA) for protein quantification.