International Journal for Parasitology xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

International Journal for Parasitology

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

Unravelling the differences: comparative proteomic analysis of a clonal virulent and an attenuated *Histomonas meleagridis* strain

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ARTICLE INFO

Article history:
Received 6 July 2017
Received in revised form 9 August 2017
Accepted 15 August 2017
Available online xxxx

Keywords:
Histomonas meleagridis
2-DE
Virulence factors
Attenuation
Histomonosis
Proteome analysis
Protozoa
Escherichia coli DH5α

ABSTRACT

The current study focused on *Histomonas meleagridis*, a unicellular protozoan, responsible for histomonosis in poultry. Recently, the occurrence of the disease increased due to the ban of effective chemotherapeutic drugs. Basic questions regarding the molecular biology, virulence mechanisms or even life cycle of the flagellate are still puzzling. In order to address some of these issues, we conducted a comparative proteomic analysis of a virulent and an attenuated *H. meleagridis* strain traced back to a single cell and propagated in vitro as monoxenic mono-eukaryotic cultures. Using two-dimensional electrophoresis (2-DE) for proteome visualization with computational 2-DE gel image and statistical analysis, upregulated proteins in either of the two *H. meleagridis* strains were detected. Statistical analysis fulfilling two criteria (≥threefold upregulation and $P < 0.05$) revealed 119 differentially expressed protein spots out of which 62 spots were noticed in gels with proteins from the virulent and 57 spots in gels with proteins from the attenuated culture. Mass spectrometric analysis of 32 protein spots upregulated in gels of the virulent strain identified 17 as *H. meleagridis*-specific. The identification revealed that these spots belonged to eight different proteins, with the majority related to cellular stress management. Two ubiquitous cellular proteins, actin and enolase, were upregulated in multiple gel positions in this strain, indicating either post-translational modification or truncation, or even both. Additionally, a known virulence factor named legumain cysteine peptidase was also detected. In contrast to this, mass spectrometric analysis of 49 protein spots, upregulated in gels of the attenuated strain, singled out 32 spots as specific for the flagellate. These spots were shown to correspond to 24 different proteins that reflect the increased metabolism, in vitro adaptation of the parasite, and amoeboid morphology. In addition to *H. meleagridis* proteins, the analysis identified differential expression of *Escherichia coli* DH5α proteins that could have been influenced by the co-cultivated *H. meleagridis* strain, indicating a reciprocal interaction of these two organisms during monoxenic cultivation.

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1. Introduction

Histomonas meleagridis is a unicellular flagellated microaerophilic protozoan of the order Tritrichomonadida (Cepicka et al., 2010). The parasite causes histomonosis (syn. blackhead, infectious enterohepatitis, histomoniasis), a globally distributed poultry disease that was responsible for the devastation of turkey industry in the USA at the end of the 19th century (reviewed in McDougald, 2005; reviewed in Liebhart et al., 2017). In recent years the disease reappeared in the European Union and USA following amendments in drug legislation due to public health concerns (Hess et al., 2015). The disastrous nature of *H. meleagridis* can be seen in turkeys, in which the parasite causes ulceration and expansion of the caeca, which together with severe liver necrosis can lead to high mortality of up to 100% (reviewed in McDougald, 2005). In chickens, the disease is less severe and the infection is usually limited to the caeca (Hess et al., 2015; Hess, 2017). In layer chickens the parasite is responsible for a substantial decrease in egg production (Esquenet et al., 2003; Liebhart et al., 2013). Until now, only a prototype live vaccine based upon an in vitro attenuated virulent strain was proven to be effective against *H. meleagridis* (Liebhart et al., 2017). Nevertheless, vaccination is not yet a commercially available option and although novel strategies are needed, the molecular repertoire of the attenuated strain and its predecessor virulent form are not understood.

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https://doi.org/10.1016/j.ijpara.2017.08.017
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Histomonas meleagridis can be propagated in vitro only in the presence of bacteria, mainly in a so-called xenic culture, which generally contains poultry-specific caecal bacteria in addition to the protozoan (Hauck et al., 2010). Routinely the *H. meleagridis* culture is established from a clinical case by inoculating the intestinal content of a bird suffering from histomonosis into a suitable medium. As such cultures often contain a mix of protozoa, further refinement by means of "mono-eukaryotic" culture, with only a single protozoan species, is much better defined (Hess et al., 2015). The establishment of such cultures can be accomplished by a micromanipulation approach and enables traceability back to a single cell (Hess et al., 2006). Even though the protozoan content of the culture is defined, the xenic mono-eukaryotic culture still contains the complexity given by the unknown bacterial content. This obstacle was circumvented by exchanging the caecal bacterial mix with a single bacterial strain, *Escherichia coli* DH5α, to establish a "monoxenic", "mono-eukaryotic" culture (Ganas et al., 2012). The attenuation of the protozoa is accomplished by extended in vitro cultivation, which was confirmed by animal experiments in chickens and turkeys (Sulejmanovic et al., 2013). This seems to be an exclusive property of the protozoan since the process of monoxenization with *E. coli* DH5α was shown to have no influence in vivo, neither on the virulent nor on the attenuated strain (Ganas et al., 2012).

Molecular understanding and characterization of *H. meleagridis* is very limited. The first molecular study reported three genes encoding for proteins that take part in essential metabolic processes and are localized in electron-dense organelles called hydrogenosomes, known as the centre of anaerobic energy metabolism (Mazet et al., 2008). A phage display study, using a *H. meleagridis* expression cDNA library, identified a wide range of partial protein coding sequences with homology to both intracellular and surface proteins (Bilic et al., 2009). The majority of these sequences were shown to be homologous to *Trichomonas vaginalis* genes, indicating that in a variety of cell processes *H. meleagridis* might be very similar to this sexually transmitted human parasite (Bilic et al., 2009). This was confirmed by sequencing a cDNA library from xenically cultivated *H. meleagridis* in which 3,425 unique contigs were identified, with the majority being homologous to *T. vaginalis* genes (Klodnicki et al., 2013). Additionally, in another study three genes encoding α-actinins were further characterized, showing for the first time the presence of three different α-actinin proteins within one protozoan and their immunogenicity in chickens and turkeys (Leberl et al., 2010). However, despite recent aggregation of sequence data, studies on the proteome are almost non-existent. The only available non-comparative proteomic analysis, which involved two-dimensional electrophoresis (2-DE), identified 19 *H. meleagridis* protein spots, of which 17 were characterized as actin (Pham et al., 2016).

In an attempt to fill the knowledge gaps in the *H. meleagridis* protein repertoire and molecular biology, the present study aimed to identify differentially expressed proteins between in vitro cultivated virulent and attenuated strains. Towards this, we performed a detailed comparative proteomic analysis of the two in vivo well-defined *H. meleagridis* strains using 2-DE for proteome visualization, combined with sophisticated computational tools for detection of significantly differentially abundant protein spots, and mass spectrometry for protein identification. 2-DE with immobilized pH gradient (IPG) strips is currently an irreplaceable technique as it can separate numerous proteins from complex samples based on their isoelectric point (pI) and molecular mass (M_r) (Görg et al., 2004). This technique, in association with mass spectrometry, was chosen in *T. vaginalis* studies, which aimed to identify changes in the protein expression between high- and low-virulence isolates (Cuervo et al., 2008; De Jesus et al., 2009).

2. Materials and methods

2.1. Cultivation of virulent and attenuated H. meleagridis parasites

Virulent and attenuated *H. meleagridis* maintained as monoxenic mono-eukaryotic cultures, designated *H. meleagridis/turkey/Austria/2922-C6/04-10x/DH5α* and *H. meleagridis/turkey/Austria/2922-C6/04-290x/DH5α*, respectively, were used for the experiments (Ganas et al., 2012). Parasites were passaged every 3 days, as previously described (Ganas et al., 2012). The cultures were incubated at 41 °C in standard medium consisting of Medium 199 (Gibco™, Vienna, Austria), 15% heat-inactivated foetal bovine serum (FBS) (Gibco™), 0.25% of sterilized rice starch (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) with the addition of 1 ml overnight *E. coli* DH5α liquid culture (colony forming units (CFU): 5×10^8 bacterial cells/ml). The liquid *E. coli* DH5α culture was incubated overnight at 37 °C in the above standard medium. For all experiments, the virulent parasites were harvested at passage number 20 (20p) and the attenuated ones at passage number 303 (303p).

2.2. Purification of H. meleagridis cultures

Parasites were harvested and purified in order to remove most of the bacteria present in the culture and parasite debris. Briefly, the cultures were transferred in 50 ml conical tubes (Sarstedt, Wiener Neudorf, Austria) and histomonads were collected by centrifugation at 200 g for 5 min at room temperature. The *E. coli* DH5α-rich supernatant was discarded and the pellet, consisting of histomonads and rice starch, was re-suspended in 15 ml of pre-warmed Medium 199. This step was repeated three times. Subsequently, the pellet was re-suspended in 2 ml of Medium 199 and transferred to 2 ml Eppendorf tubes. The quality of purification and the number of *H. meleagridis* cells were evaluated microscopically using Trypan Blue Stain (Gibco™, Invitrogen, Vienna, Austria). Finally, histomonads were pelleted at 13,000 rpm (Biofuge pico Heraeus, Kendro Laboratory Products, Hanau, Germany) for 2 min at room temperature and after removal of the supernatant the samples were immediately frozen at −80 °C until further use.

2.3. Protein extraction and sample preparation

Protein extraction was performed using similar numbers of virulent (20p) and attenuated (303p) parasites, which were cultivated in parallel and harvested on the same day. Two Eppendorf tubes with purified virulent or attenuated parasites were used for protein extraction on day one (PR1) and on day two (PR2), and as a result four protein samples were obtained. The two biological replicates from each strain (e.g. 20p-PR1 and 20p-PR2) were analysed twice and the acquired gels represented technical replicates for the 2-DE protocol (e.g. 20p-PR1 1st and 20p-PR1 2nd). Parasite pellets were resuspended in extraction buffer (50 mM Tris–HCl pH 8.8, 5 mM EDTA, 100 mM KCl, 1% (w/v) DTT and complete protease inhibitor cocktail (Roche Applied Science, Roche Diagnostics, Mannheim, Germany)). The parasites were then sonicated (power: 30%, duration: 10 s, cycle (pulsation): $5 \times 10\%$, Bandelin Sonopuls HD2070, Bandelin electronic, Berlin, Germany) three times on ice with a 30 s rest period in between.

Subsequently, the lysate was centrifuged at 18,000 *g* for 15 min at 4 °C and the pellet containing rice starch particles was discarded. Rice starch increased sample viscosity, a phenomenon that can occur during 2-DE sample preparation in the presence of polysaccharides (Görg et al., 2004). The supernatant was then mixed with 2-DE lysis buffer (pH: 8.5) containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 30 mM Tris–HCl (Arnal et al., 2015)

Please cite this article in press as: Monoyios, A., et al. Unravelling the differences: comparative proteomic analysis of a clonal virulent and an attenuated *Histomonas meleagridis* strain. Int. J. Parasitol. (2017), https://doi.org/10.1016/j.ijpara.2017.08.017

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