Interplay of hypoxia and host cells in the translocation, invasion and dissemination of *Taenia solium* in neurocysticercosis

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Abstract

Neurocysticercosis, caused by the invasion of the central nervous system (CNS) by *Taenia solium* larvae, poses a significant global health burden. Despite its prevalence and severe neurological consequences, understanding of the molecular and signalling pathways facilitating parasite dissemination and CNS invasion is limited. The lack of comprehensive knowledge of host-parasite interactions and associated proteins involved in *T. solium* infection hinders the development of targeted interventions to mitigate its ability to cross the epithelia barrier. This is complicated by reduced oxygen availability in the intestine, a phenomenon called *hypoxia.* Hypoxia can result in epithelial barrier disruption and cell damage, thereby promoting the translocation and dissemination of *T. solium*. This study aims to establish the role of hypoxia in *T. solium* invasion and disseminated infections. The effect of hypoxia on the migration, viability and morphological characteristics of *T. solium* would be determined using transwell invasion assays, flow cytometry and microscopy. *T. solium* oncosphere development and dissemination under hypoxic and normoxic conditions will be monitored using animal models. Also, host-parasite transcriptome and proteome profiling will be performed to determine pathways triggered under hypoxic conditions. It is expected that hypoxia would promote the invasion and dissemination of *T. solium* by enhancing epithelial and endothelial cell permeability. , hypoxia will induce the expression of binding and adhesion proteins and other virulence markers such as enolase, serpin, and glutathione transferases that are involved in host invasion. Understanding the role of hypoxia in the translocation mechanism of *T. solium* can be leveraged to provide insights into host tissues dissemination and the development of appropriate interventions.

Keywords

Taenia solium, Neurocysticercosis, invasion, hypoxia, virulence

Introduction

T. solium infection and its resulting disease, cysticercosis is emerging as a major public health concern. The disease is common in least-developed regions where pigs are raised for food. Although cysticercosis is a neglected tropical disease, it is increasingly spreading to non-endemic developed countries in Western Europe due to immigration and tourism (Laranjo-González M. et al. 2017). A major complication of *T. solium* infection is neurocysticercosis (NCC), which occurs when the parasite migrates to the brain. About 2.5 million people have *T. solium* infection globally, with a 50,000 annual mortality rate due to NCC (Aung and Spelman 2016).

T. solium infections occur primarily in human hosts when raw or undercooked pork infected with the parasites' cysticerci is ingested. The cysticercus develops into an adult tapeworm in the gut and attaches to the mucosal surface of small intestines (Fig. 1). Infections with the adult tapeworm are typically asymptomatic; however sometimes present with mild symptoms such as abdominal pain and diarrhoea. *T. solium* eggs (oncospheres) occasionally cross the intestinal barriers to the bloodstream and disseminate to other host tissues/organs such as the heart, brain and the orbital space (Kobayashi et al. 2013) where they develop into larval forms (cysticerci).

The cysticercus primarily targets the CNS to cause NCC, which develops approximately 1-5 years after infection with cysticerci (Garcia et al. 2018). Although NCC is the most common cause of acquired epilepsy in adults (Reddy and Volkmer 2017), there is limited information on why the cysticercus majorly targets the CNS and how it successfully invades and establishes NCC. Most studies have focused on diagnostics, clinical manifestations, and treatment of NCC. However, understanding the parasite's biology, translocation pathways, and its interaction with host factors is essential for the development of appropriate interventions for disease control.

Parasite invasion and subsequent translocation to the brain involve several mechanisms including secretion of proteolytic enzymes, disruption of the cell membrane, cell signalling pathways, and evasion of the immune system. Cysticerci invasion and subsequent dissemination require breaching the epithelial barrier and enhancing its permeability. However, there is a paucity of data on the effect of *T. solium* or its excretory/secretory products on epithelial barrier function.

An emerging factor that is implicated in epithelial barrier function is hypoxia, a state of oxygen deficiency in tissues. The oxygen level of the gastrointestinal tract is tightly regulated, decreasing in concentration from the sub-mucosa to the lumen, which results in a slightly hypoxic condition in the lumen (Singhal and Shah 2020). In host tissues, oxygen levels could fluctuate between hypoxic and normoxic states depending on the tissue's metabolic activity or pathological state (Zheng et al. 2015). Notably, the brain, which is the preferential site of the cysticercus is highly oxygenated; this is critical for its function and state. However, parasite invasion could impair blood flow, resulting in a pathological

hypoxic state such as ischemia. This pathological state could impair epithelial barrier function and parasite invasion.

The adaptation of adult *T. solium* to the guts and the cysticercus to the brain suggests that the different stages are adapted to thrive in both low (33 mmHg in the small intestine) and high (90 mmHg in the brain) oxygen levels. However, there is limited information on the factors that allow the parasite to thrive at different oxygen levels as well as how hypoxia moderates *T. solium* infection and CNS invasion. This study aims to provide insights into the role of hypoxia in the translocation, dissemination and invasion of *T. solium* in cysticercosis.

Rationale

T. solium disease manifests in different forms in the host: as small intestine taeniasis, blood and tissue cysticercosis, and neurocysticercosis in the central nervous system. Interventions for disease control would include identifying factors promoting disease progression of these various forms. Also, the complex life cycle of the parasite as well as its host adaptation suggests the involvement of specific parasitic factors at the different stages in disease pathogenesis.

However, the mechanisms that promote host tissues and CNS invasion are unclear. It is possible that intestinal hypoxia could facilitate *T. solium* cellular invasion. Also, severe hypoxia has been associated with loss of barrier function and could encourage disseminated infections and parasite translocation across epithelial cells to the brain. Moreover, intestinal protozoan parasites that invade the intestinal epithelia activate hypoxic response genes including virulent factors. Therefore, *T. solium* adaptation to the small intestines under hypoxic conditions could suggest a likely role of hypoxia in cysticerci invasion and consequently cysticercosis/neurocysticercosis development.

Research questions

1. Does intestinal hypoxia and ischemia promote *T. solium* translocation through the epithelial barrier?

2. How does this translocation pathway contribute to parasite dissemination and the development of neurocysticercosis?

3. Are there specific markers regulating the translocation of *T. solium* under hypoxic conditions?

4. What are the host's responses to *T. solium* under hypoxic conditions?

Aim

To investigate the role of hypoxia in regulating host and parasitic factors during the translocation, invasion and dissemination of *T. solium* in neurocysticercosis.

Objectives

1. To determine the translocation pathway of *T. solium* across the intestinal epithelia to the brain during normoxic and hypoxic conditions.

2.To determine the influence of hypoxia on the expression of virulence-associated genes in *T. solium.*

3. To determine host response to *T. solium* infections during normoxic and hypoxic conditions.

Data analysis

Three independent experiments will be done in triplicates. The rate of oncosphere invasion under hypoxic states and controls will be determined by quantification of viable transmigrated oncospheres and plotted against the time of exposure. For *in vivo* assays, the quantity of migrated oncospheres and affected tissues will be plotted against time. Electron micrographs of motile oncospheres and their morphological characteristics will be processed and analysed using Fiji (v2.9.0).

R software for statistical analysis, version R-4.3.1 will be used for all statistical analysis. Statistical differences between the expression profiles will be determined using ANOVA and t-tests. A *p*-value of <0.05 will be considered as significant. For RNA-seq analysis, sequenced reads will be aligned to the *T. solium* reference genome, GCA_001870725.1. The DEseq analysis pipeline will be used to identify differentially expressed genes. GoSeq will be used for gene ontology and enrichment to identify relevant pathways. Also, the FlowJo (v10.9) software package will be used to quantify immune cell infiltrates from the flow cytometry analysis

Expected outcome

This study is expected to identify the role of hypoxia in the pathogenesis and invasion of *T. solium* in cysticercosis/neurocysticercosis. It is expected that hypoxia would promote the translocation and dissemination of *T. solium* into tissues and the CNS by: i) enhancing the permeability of intestinal epithelial and blood-brain barrier and ii) promoting structural morphological changes in oncospheres that allow for motility and invasion. Hypoxia is expected to trigger host pathways such as the hypoxia-inducible factor pathway (HIF)

which will induce the expression of hypoxia related genes. , severe hypoxic conditions are expected to cause a decrease in the expression of tight junction associated protein; a major hallmark of hypoxia.

Impact

The study will provide insights into the pathogenesis, invasion and molecular mechanism involved in the translocation of *T. solium* through the intestinal and endothelial barriers. This knowledge could form the basis for the development of novel prophylactic or therapeutics, reveal potential biomarkers for risk assessment, and early diagnosis, and inform treatment strategies.

Implementation

Ethical approval

Ethical approval for animal use will be obtained from the Noguchi Memorial Institute for Medical Research Institutional Review Board (NMIMR-IRB), located within the University of Ghana. We will establish a collaboration with the Infectious disease Research Laboratory, Universidad Peruana Cayetano Heredia to source the *T. solium* eggs. Outbred Holtzman albino rats 10 to 26 days old, as previously indicated (Sitali et al. 2022) will be purchased from Taconic Biosciences and housed at the animal experimentation unit at the NMIMR. A total number of 75 rats (5 per group) (Table 1) will be used for this study as determined by the resource equation approach (Arifin and Zahiruddin 2017). The animals will be used for '*Animal housing*' and experiments will be performed under strict adherence to protocols approved for animal care and use.

Oncosphere hatching and activation

The oncospheres will be hatched *in vitro* by incubating in 0.75% sodium hypochlorite adjusted in saline for 10 min at 4°C. Subsequently, the oncospheres will be activated with artificial intestinal fluid containing 10% trypsin, 0.2% anhydrous sodium, 1% pancreatin, 1% carbonate and 0.5% pig bile in RPMI-1640 (Gibco) at 37°C with shaking (60 rpm) for 1 h. Oncospheres viability would be determined using trypan blue staining and light microscopy (100x).

Cell culture

Human intestinal epithelial cell lines (HIEC-6 - CRL-3266) and Blood-Brain Barrier hCMEC/D3 cell lines would be purchased from ATCC (USA) and MERK respectively. The HIEC-6 cell lines will be cultured in RPMI 1640 medium (Gibco™) supplemented with 10% fetal bovine serum (FBS) (Gibco™) and incubated at 37°C in a humidified atmosphere containing 5% CO_2. The hCMEC/D3 cell lines will be cultured in an endothelial basal medium (Gibco) supplemented with 5% FBS, chemically defined lipid concentrate (ADCF

Lipid Supplement, Cytiva), ascorbic acid (5 µg/mL), human basic fibroblast growth factor (1 ng/mL), and hydrocortisone (1.4 µM).

Establishment of intestinal and endothelial monolayer culture

To establish a monolayer of intestinal and endothelial cell lines, a transwell membrane cell culture inserts (3 µm pore size, Corning™) will be coated with liquefied matrigel (BD Matrigel™ Basement Membrane Matrix) and solidified into a thin gel layer, which acts as an extracellular matrix (ECM). Subsequently, single-cell suspension of HIEC-6 and hCMEC/D3 cell lines will be prepared from cultures and diluted to 1x10^6 cells/ml. A hundred microlitres (100 µl) of HIEC-6 and hCMEC/D cell suspensions would be seeded into separate matrigel-coated membrane inserts and incubated at 37°C (5% CO_2) for 10 min.

Transwell oncosphere invasion assay under hypoxic conditions

The transwell culture inserts (containing intestinal and endothelial monolayer) will be placed in tissue culture plates containing RPMI medium and inoculated with 2500 viable oncospheres suspended in normal saline. Subsequently, the cultures would be divided into three groups: (i) two hypoxic groups at 0.2%, 0.5%, 1%, 2%, 3%, and 5% oxygen levels, and (ii) the third group at 20.9% normal oxygen levels, serving as a control (McKeown, 2014). The cultures would be incubated at 37°C in a Multi Gas Incubator. The cells would be monitored for 48 h at 4 h intervals and the number of oncospheres invading the membrane would be quantified at different time points.

Flourescence microscopy

The viability of the translocated oncospheres under hypoxic and controlled conditions will be determined using flourescence microscopy. Briefly, single-cell suspension of oncospheres from the various hypoxic conditions will be stained with calcein-AM and propidium iodide dye and analyzed with a flourescence microscope (100x).

Transmission electron microscopy

The morphological characteristics of oncospheres under hypoxia as compared to control (normoxia) would be determined using Transmission electron microscopy (TEM). The oncospheres would be harvested and fixed with 1% glutaraldehyde in phosphate-buffered saline (PBS) and in 1.5% osmium tetroxide. The cells will be dehydrated in ethanol, embedded in epoxy, and sectioned to 50 nm. The sections will be stained with 3% lead hydroxide and 5% uranyl acetate and observed under the electron microscope (Philips CM10 Electron Microscope) at 75 kV. The effect of hypoxia on motility and oncosphere structures (scolex, rostellum, and cuticle) will be determined. Oncosphere motility would be defined by a frequent cyclic movement of hooks. Structurally, oncospheres would be compared in terms of size, number of visible embryonic hooklets, number of nuclei, presence of secretory vesicles, and microvilli.

Generation of a transgenic actin::nanoluc expressing *T. solium* oncosphere

To monitor cysticerci dissemination and development *in vivo*, mutant oncospheres expressing nanoluc-tagged actin will be generated using CRISPR/cas9 mediated knock-in. Briefly, the CHOPCHOP web interface (http://chopchop.cbu.uib.no) will be used to select target sites and design guide RNAs (gRNAs). The gRNAs will be designed to target the 5′ and 3′ flanking regions of the gene of interest (actin). The donor plasmid will be designed to have an actin:nanoluc Caspase9 gene fusion cassette, which will generate a glow-type signal in the presence of nanoluc luciferase. The *T. solium* oncospheres will be transfected with donor plasmids and gRNA using electroporation. PCR will be used to confirm the insertion of the actin:nanoluc Caspase9 gene fusion. All plasmids will be custom synthesized by Dharmacon (USA).

Infection of rats with transgenic *T. solium* oncospheres

To determine the effect of hypoxia on parasite dissemination, 15 days old outbred Holtzman albino rats (Verastegui et al. 2015) will be infected with transgenic *T. solium* oncospheres expressing actin::nanoluc fusion protein. Infection with transgenic oncospheres will be performed by intravenous injection. The rats will be housed in a controlled hypoxia chamber at different oxygen levels (10%, 12%, 15%, 18% and 20.9%). Oncosphere migration, invasion, and cyst development will be tracked using a Magnetic Resonance Imaging (MRI) scan at 5-day intervals for three months. Subsequently, the rats will be euthanized, and invaded tissues and organs will be harvested for histopathological analysis (Mejia Maza et al. 2019). Histopathological features such as fibrosis around the parasites and other cellular alterations will be examined using a light microscope (100x).

***In silico* identification of parasitic virulence markers**

Using the WormBase and the NCBI database, the genome of *T. solium* will be explored for the presence of parasite-associated virulence markers. These markers would include genes that have been implicated in tissue invasion in helminthes (Table 2).

Expression profile of virulence markers

The expression profile of selected virulence markers will be determined under hypoxic and normoxic states. Briefly, following the transwell invasion assay described in objective above, RNA will be extracted from invaded oncospheres under the various hypoxic conditions and controls using the QIAGEN RNA extraction kits (RNeasy kits), according to the manufacturer's instructions. Subsequently, RT-qPCR will be performed to determine the expression of virulence markers under a hypoxic state relative to the control.

RNA sequencing

To determine other differentially expressed genes and molecular pathways that are triggered in *T. solium* during hypoxia-mediated invasion, an RNA-seq will be performed. Briefly, total RNA will be extracted from oncospheres following exposure to hypoxic and normoxic conditions. An RNA library will be prepared from the total RNA using the Illumina® Stranded Total RNA Prep. The samples will be sequenced using the Illumina

RNA-seq platform and data analyzed with bioinformatics tools such as HISAT2, GSNAP and DESeq.

Proteomics

To characterize the excretory/secretory proteins that are expressed by *T. solium* under hypoxia and normoxia conditions, the tegumental surface proteins of oncospheres under hypoxic and normoxic state will be isolated as described by Torre-Escudero and Robinson (2020). Briefly, following the transwell migration assay, the culture medium will be collected and centrifuged (300 x g) at 4°C for 10 min to remove eggs and debris. Subsequently, cold acetone (absolute) would be added to the samples and incubated at -20°C for 1 h followed by centrifugation at 13,000 x g for 10 min. The pellets (containing proteins) are dried and resuspended in a buffer. The isolated protein will be subjected to SDS-PAGE (10% acrylamide gel) analysis, followed by mass spectrometry for quantification and characterization.

Generation of mutant oncospheres

To determine the significance of the virulence markers to parasite invasion under a hypoxic state, the selected virulence markers (Table 2) will be knocked out using CRISPR/cas9 as previously described.

Determination of invasive potentials of mutant oncospheres

To determine the ability of the mutant oncospheres to invade host tissues, mutant oncospheres will be subjected to migration assays under hypoxic and normoxic conditions and characterized as previously described.

Host transcriptomics

To determine host responses to *T. solium* infection under hypoxic and normoxic conditions, host transcriptome profiling will be performed using RNA-seq. Total RNA will be extracted from tissues that harbour cysticerci in infected rats and sequenced as previously described.

Profiling of immune cell infiltrates (Flow cytometry)

Immune cell infiltrates in infected tissues under hypoxic and normoxic conditions will be profiled using flow cytometry. Briefly, tissue sections harvested from rats will be minced and chemically dissociated in collagenase to obtain single-cell suspensions. The cell suspensions will be filtered using 40 µm filters, and centrifuged at 300 x g for 5 min. The cells will be washed and suspended in flow cytometry staining buffer (Invitrogen™ eBioscience™). For brain tissues, a neural tissue dissociation kit (Miltenyi Biotec) will be used to obtain single-cell suspensions. Subsequently, the cells will be stained with a customised panel of antibodies (abcam, USA) targeting immune cell markers of parasitic infection (Table 3).

Cytokine profiling

The cytokine profile of the immune cells will be determined using a multiplex cytokine bead array as described by Medeiros and Gomes (2019). Briefly, the harvested tissues will be minced, followed by the addition of an extraction buffer and electric homogenization. The samples are centrifuged at 500 x g for 10 min and the supernatants are incubated with 50 µL of bead mixture and cytokine standards for 3 h. The samples will be washed, and analysed using flow cytometry.

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Conflicts of interest

The authors have declared that no competing interests exist.

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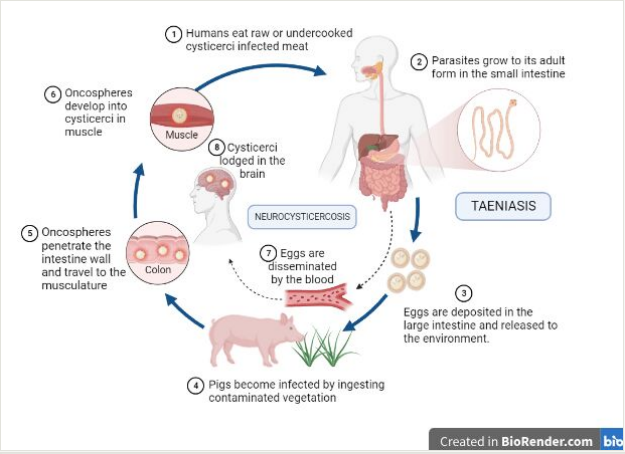

Figure 1.

The transmission route and life cycle of *T. solium*.

Table 1.

Total number of rats per each hypoxic and control groups

Route of infection	10%	12%	15%	18%	20.9% (Normoxic control)
Intravenous	5	5	5	5	5
Oral	5	5	5	5	5
No parasite infection	5	5	5	5	5

Table 2.

List of virulence genes selected for in silico analysis.

Biological processes	Genes	Function
Cytoskeleton, motility	Actin 1, Actin 2, Myophilin	Cell motility, actin binding
Proteolysis	Antigen 5	Trypsin-like protein
Signaling	Annexin A5, Anexin A6, PKB/AKT	Intracellular signaling, Division and migration
Binding, adhesion, invasion	AA6, integrin alpha	Binding and adhesion

Table 3.

List of selected immune cell markers and cytokines for flow cytometry.

	Markers
Immune cells	CD11b, CD45, CD16, CD20, CD63, CD4, CD8, CD4+CD25
Cytokines	IL-2, IL-12, IL-1ß, IL-18, IL-6, IL-13, IL-4, IL-10, TNF-α, INF-γ