International Journal for Parasitology 45 (2015) 495–503

Contents lists available at ScienceDirect

International Journal for Parasitology

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

Microarray gene expression analysis reveals major differences between *Toxocara canis* and *Toxocara cati* neurotoxocarosis and involvement of *T. canis* in lipid biosynthetic processes

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ARTICLE INFO

Article history:
Received 8 January 2015
Received in revised form 18 February 2015
Accepted 20 February 2015
Available online 3 April 2015

Keywords:
Toxocara canis
Toxocara cati
Neurotoxocarosis
Toxocarosis
Microarray analysis
Gene expression analysis
Differentially transcribed genes
Neurodegenerative disease

ABSTRACT

Toxocara canis and *Toxocara cati* are globally occurring intestinal nematodes of dogs and cats with a high zoonotic potential. Migrating larvae in the CNS of paratenic hosts, including humans, may cause neurotoxocarosis resulting in a variety of neurological symptoms. *Toxocara canis* exhibits a stronger affinity to the CNS than *T. cati*, causing more severe neurological symptoms in the mouse model. Pathomechanisms of neurotoxocarosis as well as host responses towards the respective parasite are mostly unknown. Therefore, the aim of this study was to characterise the pathogenesis at a transcriptional level using whole genome microarray expression analysis and identify differences and similarities between *T. canis*- and *T. cati*-infected brains. Microarray analysis was conducted in cerebra and cerebella of infected C57Bl/6J mice 42 days p.i. revealing more differentially transcribed genes for *T. canis*- than *T. cati*-infected brains. In cerebra and cerebella of *T. canis*-infected mice, a total of 2304 and 1954 differentially transcribed genes, respectively, were identified whereas 113 and 760 differentially transcribed genes were determined in cerebra and cerebella of *T. cati*-infected mice. Functional annotation analysis revealed major differences in host responses in terms of significantly enriched biological modules. Up-regulated genes were mainly associated with the terms "immune and defence response", "sensory perception" as well as "behaviour/taxis" retrieved from the Gene Ontology database. These observations indicate a strong immune response in both infection groups with *T. cati*-infected brains revealing less severe reactions. Down-regulated genes in *T. canis*-infected cerebra and cerebella revealed a significant enrichment for the Gene Ontology term "lipid/cholesterol biosynthetic process". Cholesterol is a highly abundant and important component in the brain, representing several functions. Disturbances of synthesis as well as concentration changes may lead to dysfunction in signal transduction and neurodegenerative disease. Overall, only a minor overlap of differentially transcribed genes was observed between the two infection groups in both brain parts. Most genes are regulated individually in each infection group, supporting the evident differences of both roundworm species observed in the paratenic host in previous studies. In summary the present study underlines the differences in pathogenicity of *T. canis* and *T. cati*. It furthermore provides a comprehensive basis for future analyses over the course of infection as well as functional tests to identify gene regulatory circuits that are crucial for pathogenesis of neurotoxocarosis. The results of this study provide a promising foundation for further specific research to evaluate the particular pathogenetic mechanisms and to identify possible therapeutic targets.

1. Introduction

Toxocara canis and *Toxocara cati* are globally occurring zoonotic roundworms of dogs and cats, respectively. The infective L3s may cause several forms of disease in paratenic hosts including humans (Magnaval et al., 2001). Besides covert toxocarosis and visceral larva migrans (VLM) with rather unspecific symptoms, ocular larva

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http://dx.doi.org/10.1016/j.ijpara.2015.02.009
0020-7519/

migrans (OLM) and the so called neurotoxocarosis play a major role in human infection. OLM is caused by larvae migrating into the eyes, causing granuloma formation, retinal damage and loss of vision (Nichols, 1956; Ashton, 1960; Glickman and Shofer, 1987). Neurotoxocarosis includes larval migration and persistence in the CNS which may lead to brain damage accompanied by symptoms such as ataxia, focal or generalised seizures, headaches, confusion, depression as well as behavioural changes in humans (Hill et al., 1985; Moreira-Silva et al., 2004; Eberhardt et al., 2005; Finsterer and Auer, 2007; Singer et al., 2011). Infective larval stages are taken up in embryonated eggs with soil, improperly cleaned vegetables and as larvae in undercooked meat (Glickman and Schantz, 1981; Stürchler et al., 1990; Dutra et al., 2014). Extensive environmental contamination with embryonated *Toxocara* eggs poses a special risk to public health. Seropositivity in humans has been reported rather frequently whereas prevalences range from 2% to 44% in Europe and 63–93% in tropical regions (Chomel et al., 1993; Magnaval et al., 1994; Uhliková and Hübner, 1998; Deutz et al., 2005; Stensvold et al., 2009). These high seroprevalences demonstrate frequent exposure of humans to infective larval stages and therefore to a high infection risk.

In mouse models, larval migrational behaviour has been studied extensively, revealing that *T. canis* exhibits a pronounced affinity to the CNS with histological changes e.g. focal malacia and demyelination, whereas *T. cati* larvae prefer visceral tissues, mainly muscle (Burren, 1971; Prokopic and Figallová, 1982; Epe et al., 1994; Cardillo et al., 2009; Janecek et al., 2014). However, if present in the brain, *T. cati* larvae are significantly more often found in the cerebellum whereat *T. canis* larvae prefer the cerebrum (Janecek et al., 2014). To date, reasons for this varying behaviour are not clear, as both species share antigenic fractions and show comparable characteristics in terms of the hepato-pulmonary migration route during the acute phase of infection (Havasiová-Reiterová et al., 1995; Fisher, 2003; Janecek et al., 2014). Even though there are described cases of *T. cati*-infected human patients (Fukae et al., 2012), most cases are assumed to be caused by *T. canis* larvae, based on larval migration patterns as well as comparable neurological symptoms in mice, resulting in an underestimation of *T. cati*-induced toxocarosis in humans (Epe et al., 1994; Fisher, 2003; Smith et al., 2009). However, diagnostic and therapeutic approaches are limited, making it difficult to differentiate between the causative agents as well as to find suitable treatment approaches.

As there is limited knowledge concerning the host response to migrating larvae and pathogenesis of neurotoxocarosis at the molecular level, the current study aimed to characterise transcriptional changes in brains of *T. canis*- and *T. cati*-infected mice via genome-wide gene expression analysis. The data will help in understanding the general changes in gene regulation during neurotoxocarosis and point out characteristic host responses to each parasite. Differences in biological and regulatory processes during the respective *Toxocara*-infection in the paratenic host can be evaluated to understand underlying molecular pathomechanisms and to detect possible targets for therapeutic approaches.

2. Materials and methods

2.1. Paratenic host model

Animal experiments were permitted by the ethics commission of the German Lower Saxony State Office for Consumer Protection and Food Safety under reference numbers 33.14-42502-04-12/0790 and 33.9-42502-05-01A038. Mice of the strain C57Bl/6JRccHsd (Harlan Laboratories, Horst, Netherlands) were used as model organisms as the microarray hybridization chips are based on the genome of C57Bl/6J mice. *Toxocara canis* and *T. cati* eggs were obtained from faeces of experimentally infected dogs and cats, respectively. Eggs were purified by a combined sedimentation-flotation method with subsequent rinsing and storage in tap water. Embryonated eggs were obtained after incubation at 25 °C for 4 weeks and stored at 4 °C until use.

Each infection group consisted of five female mice, infected orally with 2000 embryonated *T. canis* or *T. cati* eggs, respectively, in a total volume of 0.5 ml of tap water. The control group consisting of five mice was given 0.5 ml of tap water. Mice were infected at 6 weeks of age and were checked daily for general health and neurological symptoms. At day 42 p.i., mice were euthanized by cervical dislocation; brains were removed, examined for macroscopic changes and afterwards subdivided into left and right hemispheres as well as cerebrum and cerebellum. Right cerebrum and cerebellum hemispheres were stored individually in RNA*later*™ RNA stabilization reagent (Qiagen, Hilden, Germany) at 4 °C overnight and afterwards at −80 °C until RNA isolation.

2.2. RNA isolation and microarray experiment

Total RNA was isolated from cerebra and cerebella using the RNeasy® Lipid Tissue Mini kit (Qiagen) including a DNase digestion step. Isolation was carried out according to the manufacturer's instructions. Samples were eluted twice with 40 µl of RNase-free H_2O and stored in liquid nitrogen until use. Based on RNA quality control, cerebra and cerebella of three individuals were selected for microarray analysis from each infection group. Quality and integrity of total RNA were controlled using the Agilent Technologies 2100 Bioanalyzer (Agilent Technologies, Germany). Samples with a RNA integrity number (RIN) <7 were considered unsuitable for microarray analysis. A total of 100 ng of RNA were applied for Cy3-labelling according to the one-colour Quick Amp Low Input Labelling protocol (Agilent Technologies). Labelled cRNA was hybridised to Agilent's 4×44k Mouse V2, Design ID: 026655 for 17 h at 65 °C and scanned as described by Pommerenke et al. (2012). The data set has been deposited at the Gene Expression Omnibus (GEO) database of the National Center for Biotechnology Information (NCBI) under the accession number GSE66094.

2.3. Low-level analysis

Image analysis and background correction were performed using Agilent Feature Extraction 10.7.3.1 software with default parameters. Pre-processing steps consisted of background correction, scaling to mean normalisation, probe summarization and $\log_2$ transformation. Biological reproducibility of samples was evaluated by principal component analysis (PCA).

2.4. Differentially transcribed probe sets and genes (DTPs and DTGs)

Contrasts consisted of respective cerebra and cerebella obtained from each infection group and compared with the control. The selection criterion for DTPs was a fold change of $\leqslant -2.0$ or $\geqslant 2$. In addition, a P value of $\leqslant 0.05$ was chosen, which was calculated using the method of linear models for microarray data (LIMMA) embedded in Babelomics 4.3 (Medina et al., 2010). Annotations including official gene symbols of DTPs were extracted from the microarray annotation file (Agilent 4×44k Mouse V2, Design ID: 026655). Subsequently, DTPs were consolidated into DTGs by selecting the probe set with the highest significant fold change or taking transcript variants into consideration when applicable.