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Research paper

An *ex vivo* abomasal ovine model to study the immediate immune response in the context of *Haemonchus contortus* larval-stage

Saeed El-Ashram[a,b,c,*], Cuiping Li[a], Fathi Abouhajer[d], Rashid Mehmood[e], Ibrahim Al Nasr[f,g], Yinghui Zhang[h], Tang Lu[h], Ding Yili[a], Xun Suo[b], Zhang Haoji[a], Zhili Li[a], Shujian Huang[a,*]

[a] College of Life Science and Engineering, Foshan University, 18 Jiangwan Street, Foshan 528231, Guangdong Province, China
[b] State Key Laboratory for Agrobiotechnology, College of Veterinary Medicine, China Agricultural University, Beijing 100193, China
[c] Faculty of Science, Kafr ElSheikh University, Kafr ElSheikh, Egypt
[d] Faculty of Education, Asmarya University for Islamic Sciences, Zliten, Libya
[e] Department of Computer Science and Information Technology, University of Kotli, AJ&K Pakistan
[f] College of Science and Arts in Unaizah, Qassim University, Unaizah, Saudi Arabia
[g] College of Applied Health Sciences in Ar Rass, Qassim University, Ar Rass 51921, Saudi Arabia
[h] School of Food Science and Engineering, Foshan University (Northern Campus), Shishan, Naihai district of Foshan City, Guangdong Province 528231, China

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ABSTRACT

We have set up an *ex vivo* ovine abomasal model, which can mimic the multicellular process to explore the early steps in haemonchine nematode infection using RNA-seq technology. Ovine abomasal explants were collected for histological and transcriptional analysis and supernatants collected to quantitate lactate dehydrogenase (LDH) enzymes. Atotal of 233 were substantially induced genes between L_4-inoculated and uninoculated-control tissues, respectively. However, a total of 14 were considerably down-regulated genes between the 51 aforementioned tissues. Fifteen pathways were annotated by Kyoto Encyclopedia of Genes, and Genomes pathway analysis accounted for the significant percentage in immediate response to larval-stage of *H. contortus*. Key genes upregulated in response to the addition of L_4-inoculum of *H. contortus* were IL-6, IL-8, C1q, Atypical chemokine receptor-3, chemokine ligand-2, manganese superoxide dismutase, integrin alpha-7, -8, -9, integrin subunit beta-1, integrin subunit beta 6, intercellular adhesion molecule-1 and actin alpha-1. This study shows for the first time that galectin-1 is up-regulated in an *ex vivo* abomasal segment model exposed to L_4-inoculum of *H. contortus* following 6 h of incubation. The abomasal segment model has been shown to be a suitable tool to study the haemonchine larval-stage effects on the ovine abomasal tissues prior to *in vivo* assessment.

1. Introduction

Among the gastrointestinal parasites that cause losses to the farming industry, for example, *Haemonchus*, *Ostertagia*, *Trichostrongylus*, *Nematodirus* and *Cooperia*, the barber's pole worm, *Haemonchus contortus*, is the predominant, blood- sucking, highly-pathogenic, and economically-important nematode that infects small ruminants (O'Connor et al., 2006). It has been demonstrated that larvae provoked tiny haemorrhages as early as 3 days post-infection (dpi). Emergence of the larvae into the abomasal lumen commenced between 7 and 11 dpi and all worms had moulted to the 4th stage by 4 dpi. The early 4th stage (L_4) has a provisional buccal capsule, which facilitated larvae to attach to the abomasal mucosa and suck blood for the first time (Rahman and Collins, 1990). A reliable prediction and thorough understanding of the haemonchine-abomasal interactions is crucial for the parasite control. A simple cell-line model cannot imitate the physiologic milieu, especially cell–cell and cell-extracellular matrix (ECM) interactions (Rieder and Fiocchi, 2008). However, it is an essential tool for gaining insights into cellular processes in an isolated system and a supplement to *in vivo* animal experiments. While primary dissociated cell cultures permit a single-cell population to be studied, there is a clear need for exploring the crosstalk and possible feedback mechanisms where the main architecture of the abomasal tissues is preserved. *Ex vivo* tissue models are used to study many distinct biological processes and are used to minimize the use of animals (Rieder et al., 2012). Furthermore, several recent studies have reported the interaction of *ex vivo* tissue models and different parasites, such as *Schistosoma japonicum*, *Plasmodium falciparum*, *Blastocystis* spp and *Entamoeba histolytica* (Ajjampur et al., 2016; Bansal et al., 2009; Carranza-Rosales et al., 2010; Gobert et al., 2015).

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* Corresponding author at: College of Life Science and Engineering, Foshan University, 18 Jiangwan Street, Foshan 528231, Guangdong Province, China.
E-mail addresses: saeed_elashram@fosu.edu.cn (S. El-Ashram), huangshujian@fosu.edu.cn (S. Huang).

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Contemporary progresses in sequencing technologies, known as deep sequencing or next-generation sequencing (NGS) have become a comprehensive and precise tool for studying differentially expressed genes. Compared with the traditional differential screening techniques, such as real-time polymerase chain reaction and microarrays, RNA sequencing (RNA-seq) has a broader dynamic range, provides a better estimate of relative expression levels of any genomic region with higher technical reproducibility, and facilitates alternative splicing detection (Marioni et al., 2008; Yao et al., 2005). Along with these advantages, RNA-seq has also been used to reconstruct the entire organism transcriptome (Rastrojo et al., 2013; Siegel et al., 2011). High-throughput RNA-seq has played a crucial role in providing a concise view of the *L. major* promastigote stage global transcriptome (Rastrojo et al., 2013), establishing and enlightening current expression datasets, providing a solid foundation for drug discovery and vaccine development (Dillon et al., 2015), and studying the peripheral-blood mononuclear cells (PBMCs) transcriptome from *Fasciola hepatica*-infected sheep (Alvarez Rojas et al., 2016). A recent study by examined the transcriptome profiling of differentially expressed genes of *H. contortus*- infected resistant Canaria Hair Breed (CHB) and susceptible Canaria Sheep (CS) (Guo et al., 2016). To our knowledge, no studies have been reported regarding the impact of *H. contortus* larval-stage on the gene expression profiling of the ovine mucosa *in vitro*, despite the paramount importance of this parasite. For this reason, more complicated models using abomasal tissue are now being preferred to study the host-parasite interaction. Here we describe, a comprehensive analysis of the poly (A) transcriptome of *H. contortus* larval-stage treated *ex-vivo* ovine abomasal mucosal explants.

2. Materials and methods

2.1. Ethics statement

Animal experiments were conducted in accordance with the guidelines of Beijing Municipality on the Review of Welfare and Ethics of Laboratory Animals approved by the Beijing Municipality Administration Office of Laboratory Animals (BAOLA), and under the protocol (CAU-AEC-2010–0603) approved by the China Agricultural University Animal Ethics Committee. All experimental procedures were also approved by the Institutional Animal Care and Committee of China Agricultural University (The certificate of Beijing Laboratory Animal employee, ID: 15,883).

2.2. Animals and parasites

One-month-old sheeps (*Ovis aries*) that were raised under confined living conditions to avoid worm exposure were procured from a local farmer in Jin Zhan village, Chaoyang, Beijing, China. Fecal materials and blood were collected for parasitological, haemato-biochemical, and immunological analyses (unpublished data) to confirm the absence of infection. Thereby, the animals used prior to the experimental infections had no prior exposure to parasitic infections before arriving at the empirical facility. The flow chart of water-based experiments (wet lab) and computer-assisted analysis (dry lab) is shown in Fig. 1.

2.3. Ovis aries genome reference for mapping reads

In 2012, the *Ovis aries* genome was released and updated by the International Sheep Genome Consortium (ISGC). The *Ovis aries* genome is about ∼3 Gb on 27 chromosomes (26 + XY), and it encodes over 20,921 functional genes, which were previously annotated as open reading frames in the genome and led to 29,118 gene transcripts resulting from the alternative splicing of different exons in genes. The latest sheep genome release *Ovis_aries*_v3.1 (http://www.livestockgenomics.csiro.au/sheep/oar3.1.php) was employed for sequence analysis (de Simoni Gouveia et al., 2017; Wei et al., 2015).

2.4. Preparation of haemonchine larval inoculum

Infective third-stage larvae (L_3) of *H. contortus* were kindly provided by Prof. Hu from State Key Laboratory of Agricultural Microbiology, College of Veterinary Medicine, Huazhong Agricultural University, Wuhan 430070, Hubei, China. They were retained by continuous passage through parasite-free sheep. The extracted nematode eggs from ovine faecal materials using an electrical cream separator (El-Ashram et al., 2017b; El-Ashram and Suo, 2017a, b, c) were washed on a 35 μm sieve with distilled water (DW) to remove the salt. The egg number was adjusted to 500 eggs per well in 6-well cell culture plate containing DW. The plate was examined for dehydration and larval development approximately 24 h after adding the haemonchine eggs, and 10 μl of DW was added to the dehydrated well. After 24–48 h of incubation, 20 μl of the NGM (2008) nematode growth medium was added to each well of the plate. The plate was incubated for seven days at room temperature to allow parasite development to the L_3 larval-stage. The larvae were washed with sterile phosphate-buffered saline (PBS, pH 7.4) and exsheathed in 1.3% sodium hypochlorite diluted with PBS. They were then shaken for 20 min and washed with PBS 10 times (Stringfellow, 1981).

2.5. Preparation of ovine gastric content supplement

Technique for obtaining and preparing ovine gastric contents (OGCs) to be used for larval development into L_4 stage (see Preparation of media section below) has been described as follows: three 5-month-old sheep (parasite-free sheep) were slaughtered, and OGCs were strained through 4-layers of cheese cloth (Stringfellow, 1986). The filtrate was centrifuged at 12,000 rpm for 10 min and the clear supernatant sterilized by filtration (0.02 μm Millipore) and stored at −20 °C before use.

2.6. Preparation of media

Fourty ml of the medium API-1(Douvres and Malakatis, 1977) was supplemented with 1.28 ml of Fildes' reagent (a pepsin digest of defibrinated bovine blood) (Stringfellow, 1984) and 8 ml of OGC (Stringfellow, 1986). Medium API-1 with supplements had 1000 units/ml of penicillin G potassium, 1 mg/ml of streptomycin sulfate and 10 ng/ml Fungizone at pH 6.4. Approx. 50,000 larvae were developed in the above-mentioned mixture with a gas phase of 85% N_2: 5% O_2: 10% CO_2, in roller bottles rotated at 40 rev/h at 39 °C on a roller culture apparatus.

2.7. Ovine abomasal explant preparation and incubation

Immediately after slaughtering, 20 abomasal segments (2 cm^2 each) were taken aseptically from the abomasum. Obtained abomasal segments were divided into two groups, namely L_4-inoculated and uninoculated-control groups each consisting of 10 segments. Techniques for media preparation have been described by (Bansal et al., 2009; de Graaf et al., 2010) with some modifications. Hank's Balanced Salt Solution (HBSS) without phenol red (Thermo Fisher Scientific, Waltham, MA USA) at pH 6.8 was used for washing the abomasal segments (2 cm^2) of 1-month-old parasite-free sheep. Explant incubation medium was freshly prepared by adding 1.375 g D-glucose monohydrate, 500 μl gentamicin (50 mg ml^{-1}) and 5 ml amphotericin B (250 μg ml^{-1}) to 500 ml Williams' medium E at pH 6.8 (WME, containing l-glutamine without phenol red) (Thermo Fisher Scientific, Waltham, MA USA). Each explant (10 replicates) was pinned (with the submucosa facing down) onto a 4% agarose layer in Corning® tissue-culture treated culture dishes (D × H 60 mm × 15 mm) (Sigma-Aldrich, USA) containing WME with 50 larval-stages (L_4) with the above-mentioned supplements for 6 h.