

Research Note: Transcriptomic analysis of LMH cells in response to the overexpression of a hypothetical protein identified in *Eimeria tenella* SD-01 strain

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ABSTRACT Though genome sequencing of *Eimeria tenella* predicts more than 8,000 genes, the molecular functions of many proteins remain unknown. In this study, the coding region corresponding to the mature peptide of a hypothetical protein of *E. tenella* (ETH_00023950) was amplified and expressed in a bacterial system. Following preparation of polyclonal antibody that recognizes ETH_00023950, the expression of ETH_00023950 in merozoites was examined. Meanwhile, we determined the transcriptomic responses of the leghorn male hepatoma (LMH) cells to its expression. Sequencing analysis showed that one single nucleotide polymorphism and one indel of ETH_00023950 of *E. tenella* SD-01 strain were found compared with that of the UK reference Houghton strain, leading to a frame shift and a premature

stop codon. The expression of ETH_00023950 in *E. tenella* merozoites was confirmed by indirect immunofluorescence and Western blot analysis. Transcriptomic analysis showed that ETH_00023950 altered the expression of 2,680 genes (321 downregulated genes and 2,359 upregulated genes) in LMH cells. The RNA-sequencing data were consistent with the results of the quantitative real-time polymerase chain reaction (qRT-PCR). Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis revealed that differentially expressed transcripts were significantly related to 8 pathways, including oxidative phosphorylation and TGF-beta signaling pathway. These findings contribute to understanding host-pathogen interaction and secondary bacterial infections related to *E. tenella*.

Key words: *Eimeria tenella*, ETH_00023950, merozoite, expression analysis, transcriptome

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INTRODUCTION

Coccidiosis is a common intestinal infection caused by apicomplexan protozoan parasites of the genus *Eimeria*, known to cause considerable economic losses to the poultry industry (Tian et al., 2014). *Eimeria tenella*, one of the most virulent species within the genus *Eimeria*, colonizes the ceca of chicken (Tian et al., 2014). However, compared with *Toxoplasma gondii* dense granule proteins (GRAs), little is known about the roles of *E. tenella* GRAs. *T. gondii* GRA12 (TgME49_288650) was reported to be a major virulence factor associated with parasite resistance to host gamma interferon (IFN- γ) (Fox et al., 2019). Meanwhile, *T. gondii* GRA12-related genes (TgME49_308970,

TgME49_220890 and TgME49_275860) are involved in the development and persistence of chronic stage cyst burdens (Guevara et al., 2021).

In this study, the fragment of ETH_00023950 gene, sharing homology with *T. gondii* GRA12, was amplified from cDNA of *E. tenella* SD-01 strain and expressed in a bacterial system. Afterward, we prepared polyclonal antibody that recognizes ETH_00023950 and examined the expression of ETH_00023950 in *E. tenella* merozoites. Also, we examined the transcriptomic responses of the leghorn male hepatoma (LMH) cells to its expression.

MATERIALS AND METHODS

All animal protocols for this research were approved by the Animal Ethics Committee of Shanxi Agricultural University (Ethical Reference Nos. SXAU-EAW-2021C00909 and SXAU-EAW-2021M00909).

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Parasites and Cell Line

Sporulated oocysts of *E. tenella* SD-01 strain were used for total RNA isolation and complementary DNA (cDNA) synthesis. The LMH cells were cultured in Dulbecco's modified eagle medium (DMEM) plus 10% fetal bovine serum (FBS).

Construction of the Recombinant Plasmids

cDNA of *E. tenella* SD-01 strain was used as the template to amplify the coding region corresponding to the mature peptide of ETH_00023950 with the primer pair: ETH_00023950-F (5'-GGATCCGACGTTGGTATATTTTCTTCCGATG-3') and ETH_00023950-R (5'-GAATTCTCATGAGTCTCCCTTACTCCTCGCA-3'). The PCR products were double digested by restriction enzymes EcoRI and BamHI, and then ligated into pET30a vector (designated pET30a-ETH_00023950) and pCMV-N-HA vector (designated pCMV-N-HA-ETH_00023950), respectively. Validation of the recombinant plasmids was conducted through endonuclease restriction digestion and DNA sequencing (Tsingke Biotechnology Co., Ltd, Shanghai, China). Sequence alignment was performed using Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>).

Recombinant Expression of ETH_00023950 and Generation of Polyclonal Antibody

pET30a-ETH_00023950 was transformed into *Escherichia coli* BL21 and induced by isopropyl- β -D-thiogalactoside (IPTG). Following purification of the recombinant protein ETH_00023950 (rETH_00023950), its purity was evaluated by SDS-PAGE. Purified rETH_00023950 was verified by Western blot using chicken anti-*E. tenella* serum and mouse monoclonal antibody to 6X His tag, respectively. Anti-rETH_00023950 serum was generated from mice by intraperitoneal immunization as previously reported (Liu et al., 2022).

Investigation of Native ETH_00023950 Expression

An indirect immunofluorescence assay was carried out to examine the expression of ETH_00023950 in the merozoite stage of *E. tenella* as previously described (Liu et al., 2022). Briefly, merozoites were smeared onto glass slides, followed by fixation with 2% paraformaldehyde for 10 min. Then, the merozoites were treated with Triton X-100 (0.1% in PBS) for 10 min. Following incubation with mouse anti-rETH_00023950 serum and washing, the slide was incubated with goat anti-mouse IgG-FITC (Abcam, Cambridge, UK). The slide treated with the normal mouse serum was used as control. The merozoites were viewed under an inverted fluorescence microscope (Nikon, Tokyo, Japan).

For Western blot analysis, total proteins were extracted from merozoites by using RIPA lysis buffer

(Beyotime, Nantong, China). Protein extracts were separated using 10% Expressplus PAGE Gels (Genscript, Nanjing, China), and then blotted on a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA). Following blocking, the membrane was incubated with mouse anti-rETH_00023950 serum. Afterward, the membrane was hybridized with goat anti-mouse IgG-HRP (Abcam, Cambridge, UK). Detection was carried out with ECL reagent (Thermo Scientific, Waltham, MA).

Expression Analysis of the Transfected Cells

LMH cells were transfected with the pCMV-N-HA-ETH_00023950 vector and the empty pCMV-N-HA vector using Xfect Transfection Reagent (Takara, Dalian, China), respectively. The indirect immunofluorescence technique was employed to examine the transfected cells. Briefly, cells were fixed 48 h post-transfection with 2% paraformaldehyde for 10 min. After washing thrice with PBS, the cells were treated with 0.1% Triton X-100 for 10 min. Mouse anti-HA tag antibody (Abcam, Cambridge, UK) was used as the primary antibody, and goat anti-mouse IgG-FITC (Abcam, Cambridge, UK) (dilution of 1:5,000) was used as the secondary antibody. Finally, the cells were observed under an inverted fluorescence microscope (Nikon, Tokyo, Japan).

For Western blot analysis, cells were treated with RIPA lysis buffer (Beyotime, Nantong, China) 2 d post-transfection. Proteins separated by SDS-PAGE were electroblotted to a PVDF membrane. After blocking, the membrane was incubated with mouse anti-HA tag antibody for 2 h. After washing, the membrane was hybridized with goat anti-mouse IgG-HRP for 1 h. The ECL reagent (Thermo Scientific, Waltham, MA) was used to examine the chemiluminescence.

Transcriptome Sequencing and Read Alignment

The LMH cells transfected with pCMV-N-HA-ETH_00023950 or pCMV-N-HA vector were submitted to Novogene Corporation (Beijing, China) for transcriptome sequencing. Three biological replicates were performed, and isolation of total RNA was conducted by using TRIzol Reagent. The quality and concentration of the extracted RNA were assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, Palo Alto, CA). After messenger RNA (mRNA) purification using poly-T oligo-attached magnetic beads and fragmentation, first strand cDNA was synthesized. Following second strand cDNA synthesis, adapter ligation, PCR amplification and purification with AMPure XP system, the transcriptome libraries was obtained. Following cluster generation, the Illumina Novaseq platform was used for RNA sequencing. Low quality reads and reads containing adapter or ploy-N

were removed, and clean reads with high quality were aligned to the chicken (*Gallus gallus*) genome using Hisat2 v2.0.5.

Bioinformatics Analysis

Differentially expressed mRNAs between 2 groups were identified using the DESeq2 R package (1.20.0). The Benjamini-Hochberg's approach was applied to control the false discovery rate (FDR). Genes with a FDR adjusted *P* value lower than 0.05 and $|\log_2\text{FoldChange}|$ over 1.0 were assigned as differentially expressed genes (DEGs). Gene Ontology (GO) enrichment analysis was implemented by the clusterProfiler R package, and a *P* value lower than 0.05 were considered to be significantly enriched. Kyoto Encyclopedia of Genes and Genomes (KEGG) database (<http://www.genome.jp/kegg/>) was exploited for pathway analysis.

Validation of Transcriptomics Data by Quantitative Real-Time Polymerase Chain Reaction

Validation of RNA-sequencing (RNA-Seq) results was carried out by quantitative real-time polymerase chain reaction (qRT-PCR). GAPDH was selected as the endogenous control gene, and 6 DEGs from transcriptome data were used for validation. The qRT-PCR reactions were conducted in triplicates, and the relative gene expression was determined using the $2^{-\Delta\Delta CT}$ relative expression calculating method (Livak and Schmittgen, 2001).

RESULTS AND DISCUSSION

The fragment of ETH_00023950 was successfully amplified from cDNA of *E. tenella* SD-01 strain (Figure 1A). Sequencing alignment showed that the gene contains one single nucleotide polymorphism (SNP) and one insertion compared with the UK reference Houghton strain, leading to a frame shift and a premature stop codon (Figures 1B and 1C). *E. necatrix* and *E. tenella*, the 2 species of the genus *Eimeria*, are considered to be of high pathogenicity (Liu et al., 2022). Compared with *E. acervulina*, *E. maxima*, and *E. mitis*, the sequence of ETH_00023950 of *E. tenella* showed higher homology with that of *E. necatrix*, indicating that ETH_00023950 may represent a virulent factor. rETH_00023950 was confirmed by monoclonal antibody to 6X His tag (Figure 1D). Meanwhile, chicken anti-*E. tenella* serum reacted with rETH_00023950 (Figure 1E), indicating that ETH_00023950 could activate the immune system of the host and lead to an antibody response.

The asexual replicative phases are the most pathogenic part of the life cycle of *E. tenella* (Tian et al., 2014); hence, identification of genes expressed in the merozoite stage is of great importance. These findings reveal, for the first time, the expression of ETH_00023950 in merozoite stage of *E. tenella* (Figures 1F and 1G). Intriguingly, a previous study showed that another protein sharing homology with *T. gondii* GRA12 was not detected in *E. tenella* merozoites (Song et al., 2020).

Successful transfection was confirmed by indirect immunofluorescence and Western blot analysis (Figures 2A and 2B). RNA-Seq reads have been archived in the

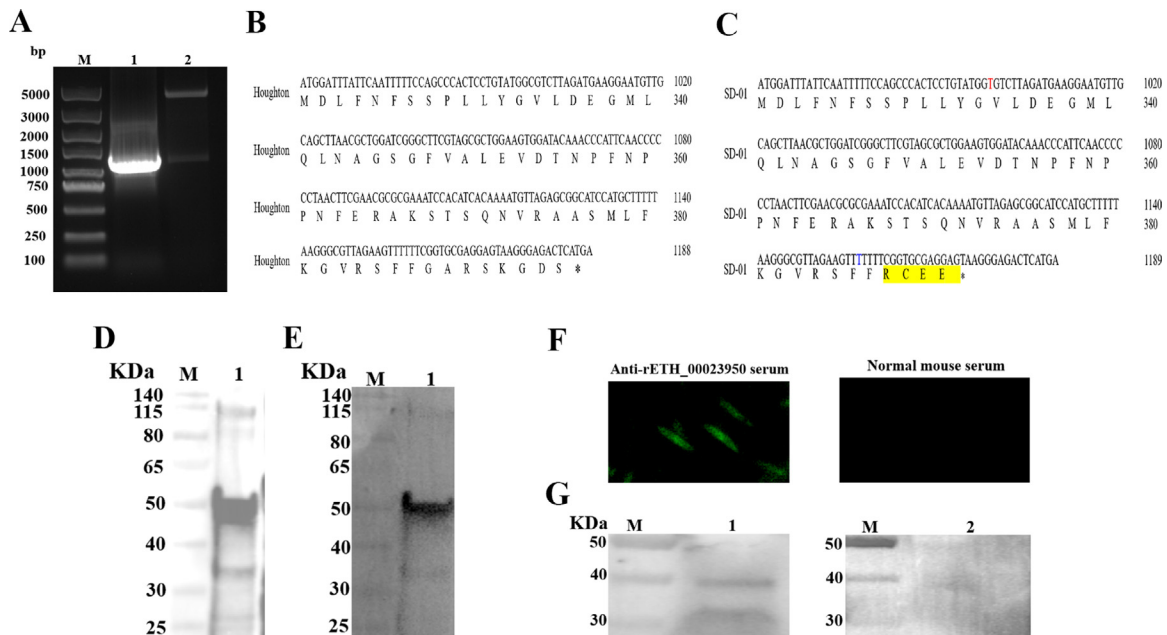


Figure 1. The gel electrophoresis of the gene fragment of ETH_00023950 (A). Nucleotide and amino acid sequences of ETH_00023950 of *E. tenella* SD-01 strain (B). Nucleotide and amino acid sequences of ETH_00023950 of the UK reference Houghton strain (C). Red: SNP; blue: indel; yellow shadow: different amino acids; *: stop codon. Western blot analysis of rETH_00023950 using monoclonal antibody to 6X His tag (D) and chicken anti-*E. tenella* serum (E), respectively. Fluorescence microscopic photographs of merozoites (F). Expression analysis of ETH_00023950 in merozoites by Western blot (G).

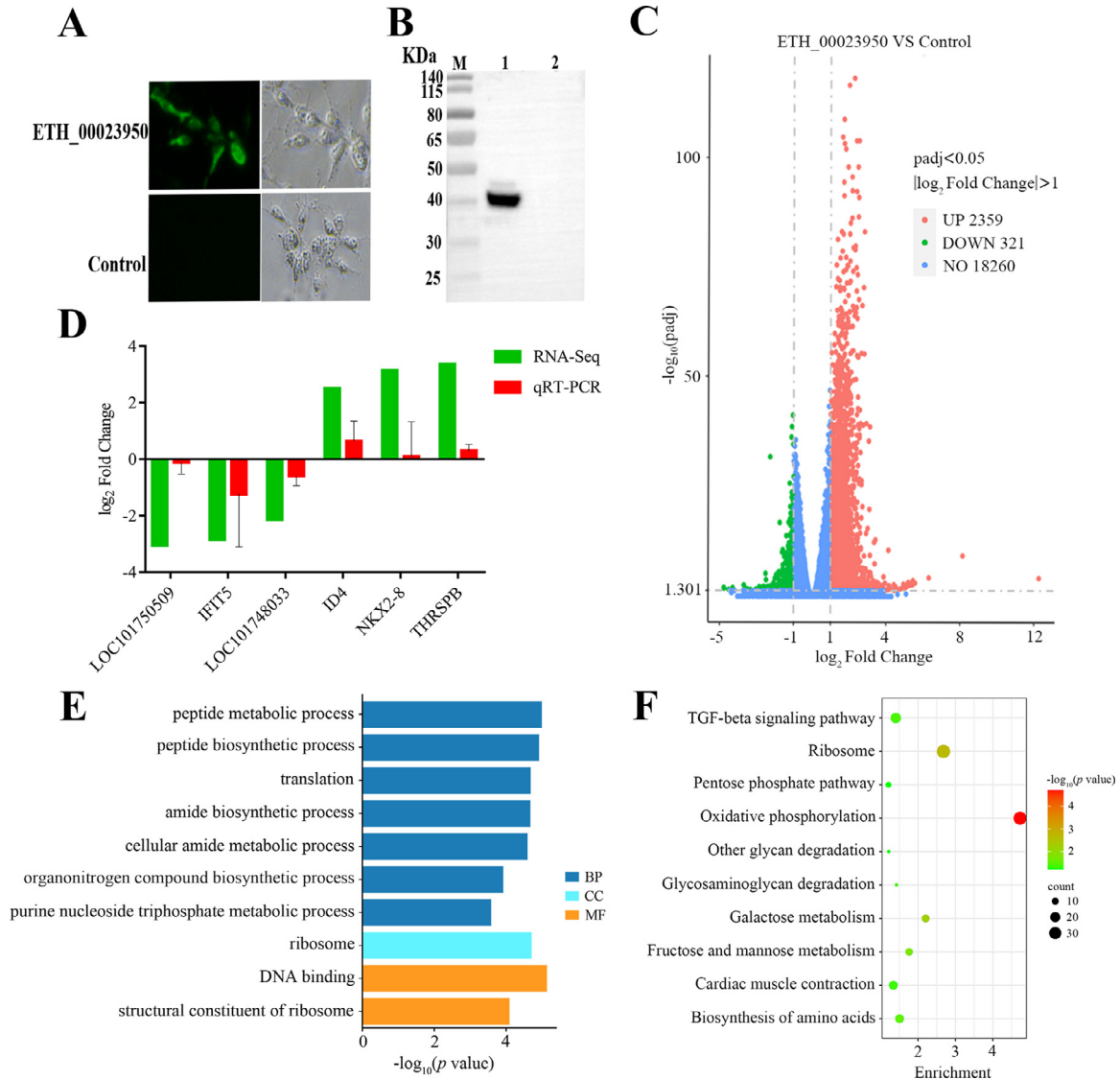


Figure 2. Immunofluorescent staining of the transfected LMH cells (A). Western blot analysis of the transfected LMH cells (B). Lane 1: protein of the pCMV-N-HA-ETH_00023950-transfected LMH cell lysate; lane 2: protein of the pCMV-N-HA-transfected LMH cell lysate. Volcano plots showing the DEGs of LMH cells transfected with pCMV-N-HA-ETH_00023950 compared to control cells (C). Six DEGs were selected for qRT-PCR validation (D). The top 10 significantly enriched GO terms (E). The eight significantly enriched pathways for DEGs (F).

NCBI sequence read archive (SRA) with the accession number PRJNA814394. Comparison of mRNA library from LMH cells transfected with pCMV-N-HA-ETH_00023950 with that of LMH cells transfected with pCMV-N-HA identified 2,680 differentially expressed transcripts, of which 2,359 were upregulated and 321 were downregulated (Figure 2C). The transcript expression patterns between two groups were verified by determining the expression of 6 DEGs by using qRT-PCR. The expression trend of the DEGs examined by qRT-PCR was consistent with that obtained by RNA-Seq analysis (Figure 2D). The DEGs were annotated into 753 GO terms, and 122 terms were found significantly enriched based on the cutoff mentioned above. The top 3 GO terms include DNA binding, peptide metabolic process and peptide biosynthetic process (Figure 2E), indicating that ETH_00023950 may be associated with cell homeostasis during *E. tenella* infection.

Pathway enrichment analysis was carried out to further determine the cellular functions altered by ETH_00023950. Using a P value < 0.05 as a cutoff, the DEGs were significantly enriched in 8 pathways (oxidative phosphorylation, ribosome, galactose metabolism, biosynthesis of amino acids, fructose and mannose metabolism, glycosaminoglycan degradation, TGF-beta signaling pathway, and cardiac muscle contraction; Figure 2F). Modulation of host cell metabolism by intracellular pathogens plays a critical role in the pathology of infection (Moreira et al., 2015). Our findings showed that *E. tenella* may affect oxidative phosphorylation of host cells using ETH_00023950, and further studies are needed to confirm this. TGF- β signaling pathway was reported to cause wide-range immune suppression (Thomas et al., 2016), which indicated that ETH_00023950 may play an important role in intracellular survival of *E. tenella*. Meanwhile, activation of

TGF- β signaling pathway was reported to increase host susceptibility to bacterial coinfection (Li et al., 2015), indicating that ETH_00023950 may be responsible for microbiome dysbiosis during *E. tenella* infection (Macdonald et al., 2017).

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DISCLOSURES

The authors declare that they have no competing interests.

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