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High-throughput sequencing reveals differing immune responses in the intestinal mucosa of two inbred lines afflicted with necrotic enteritis

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ABSTRACT

We investigated the necrotic enteritis (NE)-induced transcripts of immune-related genes in the intestinal mucosa of two highly inbred White Leghorn chicken lines, line 6.3 and line 7.2, which share the same MHC haplotype and show different levels of NE susceptibility using high-throughput RNA sequencing (RNA-Seq) technology. NE was induced by the previously described co-infection model using *Eimeria maxima* and *Clostridium perfringens*. The RNA-Seq generated over 38 million sequence reads for Marek's disease (MD)-resistant line 6.3 and over 40 million reads for the MD-susceptible line 7.2. Alignment of these sequences with the Gallus gallus genome database revealed the expression of over 29,900 gene transcripts induced by NE in these two lines, among which 7,841 genes were significantly upregulated and 2,919 genes were downregulated in line 6.3 chickens and 6,043 genes were significantly upregulated and 2,764 genes were downregulated in NE-induced line 7.2 compared with their uninfected controls. Analysis of 560 differentially expressed genes (DEGs) using the gene ontology database revealed annotations for 246 biological processes, 215 molecular functions, and 81 cellular components. Among the 53 cytokines and 96 cytokine receptors, 15 cytokines and 29 cytokine receptors were highly expressed in line 6.3, whereas the expression of 15 cytokines and 15 cytokine receptors was higher in line 7.2 than in line 6.3 (fold change ≥ 2, $p < 0.01$). In a hierarchical cluster analysis of novel mRNAs, the novel mRNA transcriptome showed higher expression in line 6.3 than in line 7.2, which is consistent with the expression profile of immune-related target genes.

In qRT-PCR and RNA-Seq analysis, all the genes examined showed similar responses to NE (correlation coefficient $R = 0.85–0.89$, $p < 0.01$) in both lines 6.3 and 7.2. This study is the first report describing NE-induced DEGs and novel transcriptomes using RNA-seq data from two inbred chicken lines showing different levels of NE susceptibility. These findings provide important insights into our current knowledge of host-pathogen interaction and the nature of host genes that can serve as NE resistance markers for molecular breeding.

1. Introduction

Necrotic enteritis (NE) is an acute or chronic enterotoxemia caused by *Clostridium perfringens* (*C. perfringens*) where a prior infection with *Eimeria maxima* (*E. maxima*) is a primary risk factor in chickens, turkeys, and ducks (Bannam et al., 2011; Savva et al., 2012; Mot et al., 2013). With the regulatory ban of antibiotic growth promoters, NE which is caused by netB toxin-producing *C. perfringens* type A and to a lesser extent by type C strains (Songer, 1996; Yan et al., 2013), is becoming an important enteric disease in chicken worldwide (Bannam et al., 2011; Savva et al., 2012; Mot et al., 2013) and has been estimated to cost the world poultry industry approximately $2 billion annually (McReynolds et al., 2004). However, the molecular mechanisms underlying the pathology of NE remain to be determined. High-throughput RNA sequencing (RNA-Seq) is a recently developed approach that uses a massively parallel sequencing strategy to generate transcriptome profiles (Wang et al., 2013) and is considered a revolutionary tool for transcriptomics, as it can absolutely quantify millions of unknown transcripts. It has also shown great analytical power for the identification of transcripts that are differentially expressed in response to different conditions (Wang et al., 2011; Xia et al., 2013).

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In a dual infection model of NE, *E. maxima* facilitates the replication and toxin production of *C. perfringens* and is thus used in conjunction with *C. perfringens* for the development of experimental NE disease models (Lee et al., 2011). To date, many studies have used RNA-seq technology for transcriptome analysis in mammals. However, this is the first time this technology has been used to study host pathogen interaction in NE in the intestinal mucosa using two inbred White Leghorn chickens differing NE susceptibility. In this study, we experimentally induced NE using *E. maxima* and *C. perfringens* to identify differentially expressed mRNA in intestinal mucosa of the chicken lines 6.3 and 7.2 which have been selected for their disparate susceptibility to Marek's Disease (Briles et al., 1977). Our new findings provide further insights into the molecular mechanisms that underline posttranscriptional regulation by mRNA of mucosal tissue in NE-afflicted chickens that will facilitate the development of novel strategies to control NE and the new genes identified in this study will be useful molecular markers for selecting NE resistance in commercial chickens.

2. Materials and methods

2.1. Experimental animals

The two White Leghorn chicken lines, namely, the MD-resistant line 6.3 and MD-susceptible line 7.2, were obtained from the Avian Disease and Oncology Laboratory (East Lansing, MI, USA) of the Agricultural Research Service of the United States Department of Agriculture (USDA). Chickens were raised in Petersime starter brooder units, provided ad libitum access to water, and maintained under uniform standard management conditions. Experimental and control groups were kept in separate rooms, preventing cross-contamination throughout the course of the experiment.

2.2. Experimental NE disease model

The method used to produce NE was previously described (Jang et al., 2012). Chickens were infected with *E. maxima* (1.0×10^4 oocysts/bird) by oral gavage on day 14, which was followed by oral gavage with a field strain of *C. perfringens* strain Del-1 (1.0×10^9 colony forming units [CFU]/bird) after 4 days. For development of NE, the birds were fed a non-medicated commercial basal ration of 17% crude protein from 1 to 18 days of age, and at 18–20 days of age, the feed was replaced with commercial non-medicated feed containing 24% crude protein. All protocols were approved by the Beltsville Area Institutional Animal Care and Use Committee.

2.3. Total RNA preparation

The small intestines from five chickens per group were cut longitudinally and briefly washed 3 times with ice-cold Hanks' balanced salt solution (HBSS) containing 100 U/mL penicillin and 100 mg/mL streptomycin (Sigma, St. Louis, MO, USA). The mucosal layer was carefully removed using a cell scraper (Nunc, Thermo Scientific Inc., Waltham, MA, USA). The samples were placed on ice immediately and maintained on ice until the total RNA extraction. Total RNA was isolated using TRizol reagent (Invitrogen, Carlsbad, CA, USA) as described (Hong et al., 2012). RNA concentrations were quantified using a NanoDrop spectrophotometer (NanoDrop Technologies, USA), and the 260/280 nm ratio was confirmed to be between 1.7 and 2.0. The integrity of the total RNA samples was evaluated using the Agilent 2100 (Agilent Technologies, Inc., Santa Clara, CA, USA) and Tecan F2000 (Tecan Group Ltd., Männedorf, Switzerland) devices, and only samples with an RNA integrity number (RIN) >7.0 and high-quality RNA (28S/18S > 1) were used for the subsequent experiments.

2.4. mRNA sequencing and analysis

Reverse transcription was performed and cDNA was synthesized using 5′ adaptor forward and 3′ adaptor reverse primers. Libraries for Illumina sequencing were constructed from cDNA as described (Trapnell et al., 2010). High-throughput RNA sequencing was performed by Theragen Bio Institute (Suwon, Korea) on an Illumina HiSeq 2000 high-throughput sequencer (Illumina, Inc. San Diego, CA, USA) according to the manufacturer's specifications. RNA-Seq data were analyzed according to the method described (Trapnell et al., 2012). Briefly, reads were mapped to the *Gallus gallus* reference genome (v.4.0) obtained from the University of California, Santa Cruz (UCSC) database (UCSC: http://genome.ucsc.edu/) using TopHat v.2.0.3 (http://tophat.cbcb.umd.edu/), and Bowtie v.0.12.8 (http://bowtie-bio.sourceforge.net/index.shtml) from Illumina iGenomes (http://support.illumina.com/). Gene expression values were measured for each gene from the Ensembl database by fragments per kilobase of exon per million mapped reads (FPKM) calculated using Cufflinks v2.0.1 (http://cufflinks.cbcb.umd.edu/) (Mortazavi et al., 2008). Differentially expressed genes were considered in a given library when (1) the *p*-value was less than 0.01 and (2) a greater-than-or-equal to 2-fold change in expression across libraries was observed and used to identify the genes differentially expressed between two chicken lines. Subsequently, the differential expression pattern analysis of known mRNA and prediction of novel mRNA were performed using unannotated sRNAs. Gene ontology (GO) terms and annotations were matched on GO terms in the database (http://www.geneontology.org/), and the functional enrichment analysis was performed using Blast2GO (v.2.7.1) (http://www.blast2go.org/). Further, significantly differed genes from the corresponding library were searched against the Kyoto Encyclopedia of Genes and Genomes (KEGG) database to determine the pathways using DAVID Bioinformatics Resources version 6.7, NIAID/NIH (http://david.abcc.ncifcrf.gov/tools.jsp) with $p < 0.01$.

2.5. Hierarchical cluster analysis for mRNA

Hierarchical cluster analysis was performed for mRNAs using Cluster version 4.49 software http://www.bram.org/serf/Clusters.php and Java Treeview software (http://sourceforge.net/projects/jtreeview/files/). Intestinal samples of lines 6.3 and 7.2 were compared as treatment and control samples, respectively. Cluster map analysis of genes was performed using the Euclidean distance. The p values were calculated using the right-tailed Fisher's exact test.

2.6. cDNA synthesis

For analysis of mRNA gene expression levels, 5 μg of total RNA was treated with 1.0 unit of DNase I and 1.0 μL of 10 × reaction buffer (Thermo Scientific, Waltham, MA, USA), and incubated for 30 min at 37 °C. Subsequently, to inactivate DNase I, 1.0 μL of 50 mM EDTA was added and the mixture was heated to 65 °C for 10 min. RNA was reverse-transcribed using the Maxima First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's recommendations. Briefly, 5.0 μg of RNA was combined with 0.2 μg of oligo $(dT)_{18}$ primer and RNase-free water to give a total volume of 12.0 μL. Then, 4.0 μl of 5 × reaction buffer, 20 units of Ribolock RNase Inhibitor, 10 mM dNTP mix, and 200 units of RevertAcid M-MuLV reverse transcriptase were added. The mixture was incubated at 42 °C for 60 min and heated at 70 °C for 5 min to terminate the reaction. After cDNA synthesis, the gene