Gene expression profiles of the small intestine of village chickens from an Ascaridia galli infested environment

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ABSTRACT

Nematodes of the genus Ascaridia are known to infect many species of birds and result in fatal diseases. A. galli damages the intestinal mucosa of chickens leading to blood loss, secondary infection and occasionally the obstruction of small intestines due to high worm burden. This study investigated the gene expression profiles in chickens from two different provinces of South Africa naturally exposed to A. galli infestations and tested either positive or negative for the parasite. The study further investigated gene expression profiles of the A. galli infected duodenum, jejunum and ileum tissues of the small intestines. The A. galli positive intestines displayed hypertrophy of the intestinal villi with accumulation of inflammatory cells and necrosis of the crypts of Lieberkühn glands, lesions that were absent in the uninfected intestines. Total RNA isolated from small intestines of infected and non-infected intestines was sequenced using Illumina HiSeq technology to generate up to 23,856,130 reads. Between any two-way comparisons of the intestines, 277 and 190 transcripts were significantly expressed in Limpopo and KwaZulu-Natal (KZN) chickens, respectively. Gene ontology analysis of the differentially expressed genes (DEGs) revealed an enrichment of genes reported to function in the immune response, defense response, inflammatory response and cell signalling genes. T cell receptor signalling pathways and arachidonic acid metabolism pathways were among the most significantly impacted pathways. Overall, the study provided insights into adaptive mechanisms for chickens extensively raised in parasite infected environments.

1. Introduction

Village chickens are raised by smallholder farmers under low-input production systems (Milieni et al., 2009), where they forage for their feed and as such are predisposed to parasites that coexist in the environment. Parasite infestation contributes to poor production and can cause mortality in severe cases (Das et al., 2010; Gauly et al., 2005; Permin et al., 2006). Infection with A. galli, that has been reported in South African village chickens (Mukaritirwa and Khumalo, 2010; Mwale and Masika, 2011), contributes to economic losses due to poorer feed conversion, reduced weight gain and decreased egg production.

Genetic control strategies have been suggested as alternatives for disease and parasite control in chicken production systems (Lamont, 1998). Genetic resistance to infection is likely as a result of heritable genes or genomic loci that have a direct or indirect role in expression of molecules that appropriately regulate host immunity to control infection and limit pathology. Variants in these genes and/or genomic loci can be applied to breeding programs to enhance parasite resistance.

The normal habitat of the parasitic stages of A. galli is in the small intestine of poultry (Soubhy, 1982). The life cycle of A. galli is direct and involves a single host (Permin et al., 1997). Mature worms live in the lumen of the small intestine, whereas eggs containing infective stage larvae (L3) develop in the environment (Permin et al., 1997). The intestine, as a first barrier, is in contact with commensal and pathogenic microorganisms (Abreu, 2010). The intestinal epithelium is therefore a critical component of a communication network that is essential for transmitting signals generated in response to infection with pathogens to cells of the innate and acquired immune systems in the underlying intestinal mucosa. The response to changes allow the intestine to contribute to the defence against pathogens and to the control and regulation of the local immune response.

Identification of tissue-specific expression signatures has both theoretical and practical implications toward understanding host–parasite interactions. Gene expression influenced by infection can be measured
on multiple tissues to give insight into the regulation of genes and pathways during infection. RNA sequencing (RNA-seq) is a high-throughput method developed for profiling transcriptomes that is cost-economic, time saving and can generate transcriptome data for non-model species (McIntyre et al., 2011; Wang et al., 2009). The approach allows gene expression profiling and in contrast to array-based techniques allows for the characterization of both known and unknown gene transcripts. Furthermore, data outputs are based on read counts instead of microarray hybridization intensity signals, which renders the technology feasible in studying variations in transcript sequences (Mortazavi et al., 2008). This study investigated the gene expression profiles of small intestines of South African village chickens with naturally acquired A. galli infestations. Furthermore, the three segments of the small intestine (duodenum, jejunum and ileum) were investigated independently in order to analyze variations of gene expression that could play a role in the site-specific functionalities of the gastro-intestinal tract. The end goal was to make inferences on genes, pathways and biological processes that play a role in resistance and tolerance to diseases and infections in village chickens exposed to gastrointestinal Nematode infestations with particular emphasis on A. galli.

2. Materials and methods

2.1. Chicken populations and intestinal tissue sample selection

A total of 144 non-descript adult village chickens were purchased from the villages in Limpopo (n = 99) and KwaZulu-Natal (KZN, n = 45) provinces. These chickens were reared under village chicken production system where no feed supplements or housing is provided and the environment is contaminated with parasites inclusive of A. galli (Malatji et al., 2016a, 2016b). The 144 chickens were euthanised by cervical disarticulation immediately after purchase. Of these 144 chickens, 40 were positive for A. galli infections, 18 of which were from Limpopo province and 22 from KZN province with worm burdens ranging from 1 to 150 worms per chicken. Intestinal tissue was sampled from chickens positive and negative for A. galli parasitism from each province. The A. galli parasites that were recovered from Limpopo and KZN chickens indicated no significant genetic differentiation (Malatji et al., 2016a) indicating that a similar population of parasites was infecting chickens between the two provinces. From each of the selected chickens, 1.5–2 cm long jejunum, ileum and duodenum tissues were collected for histological examination. Each sample was opened along the mesenteric border and placed in tissue cassettes (32 mm, L x 26 mm W, Themo Scientific) with wet filter papers to avoid curling and folding of the tissue.

2.2. Histology

A total of 86 and 97 A. galli parasites were recovered from the intestines of Limpopo and KZN positive chickens respectively. The intestinal tissue samples were fixed in 4% neutral buffered formaldehyde for 24 h and transferred to 70% ethanol. The samples were then processed conventionally, post-trimmed and embedded in paraffin. A 3 μm thick slide was made from the intestinal sample, mounted on conventional glass slides and stained with haematoxylin and eosin (HE) for overall evaluation. From every chicken two transverse sections of each intestinal sample were evaluated using light microscopy (Scale bar = 100 μm, x20).

2.3. RNA isolation from small intestine tissue

Three 60 mg tissue specimens were cut from each of the jejenum, ileum and the duodenum of the infected and non-infected chickens from KZN and Limpopo provinces. The three tissues from each section (jejenum, ileum and duodenum) of animal intestine were crushed using mortar and pestle into a homogenate from which total RNA was isolated for each section, using RNeasy Mini Kit (Qiagen), according to instructions of the manufacturer. RNA was eluted with 50 μl RNase-free water and stored at −80 °C. This was followed by a subsequent sample purification using RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. To remove contamination from genomic DNA, the RNA sample was DNase-treated using RNase-free DNase I (Qiagen). RNA concentration and quality were detected by measuring absorbance at 260 nm and A260/A280 ratio using NanoDrop (Thermo Scientific). The extent of RNA degradation was assessed by electrophoresis of 1 μg of RNA on a 1.8% agarose gel. The 12 RNA samples were stored in small aliquots at −80 °C until further use.

2.4. Sequencing using Illumina HiScan SBS technology

The TrueSeq RNA Sample Preparation kit (Illumina, San Diego, CA) was used for mRNA library preparations following the manufacturer’s protocol. Agilent 2100 Bioanalyzer was used to monitor quality control on the samples. Sequencing was performed using Illumina HiSeq 2500 at the Agricultural Research Council-Biotechnology Platform, South Africa to generate 125bp paired-end data.

2.5. Quality control of sequence data

Quality control was first performed on the primary sequencing data produced by Illumina HiSeq 2500. Sequencing adapters were trimmed off from the reads. For each of the sequencing reads, low quality bases with quality score greater than 20 as determined by phred (Ewing and Green, 1998; Ewing et al., 1998) along 95% of the read length were also trimmed using CLC Bio workbench version 6.5 (CLC Bio, Aarhus, Denmark). Reads that were > 20 bp long on both sides of paired-end format were kept for further downstream analysis and were classified as high-quality reads.

2.6. Mapping of RNA-Seq reads to reference genome

Quality controlled reads were aligned to the gallus.gallus 4.74 reference genome using TopHat v2.0.11 (Trapnell et al., 2009). TopHat uses Bowtie v2.1.0 (Langmead et al., 2009) to perform the alignment based on quality information accompanying each read, an initial alignment to the reference genome using default parameters. TopHat allowed a maximum of two mismatches when mapping reads to the reference and binary alignment map (BAM) files containing mapped and unmapped reads with a mapping report were produced.

2.7. Data preparation for transcript assembly and differential gene expression

Reads were assembled into transcripts using the default parameters of Cufflinks (Trapnell et al., 2012). The abundance of assembled transcripts was estimated and reported as fragments per kilobase of exon per million fragments mapped (FPKM), with confidence intervals estimated for each FPKM and filtering cut-off of 1.0. The DEGs ratios were tested for statistical significance using Bayesian statistics as described by Bullard et al. (2010). The significance scores were corrected for multiple testing using the Benjamini-Hochberg correction (Benjamini and Hochberg, 1995). DEGs were defined as those with absolute value of log2 fold change higher than two and P-value was less than 0.05. DEGs for the whole small intestine and three regions of the small intestines were calculated using the ratio of A. galli infested versus normal FPKM values for every gene. Genes with expression level fold change between two conditions of more than 2, p < 0.05 and False discovery rate (FDR) < 0.05 were considered DEGs. Cufflinks constructed a minimum set of transcripts that best described the reads in the generated dataset. It also used the normalized RNA-Seq fragment counts to measure the relative abundances of transcripts. The unit of
Cuffdiff was used to find significant changes in transcript expression, splicing, coding output and promoter use (Trapnell et al., 2012). It used the Cufflinks transcript quantification module to calculate transcript/gene expression levels and test them for significant changes. Cuffdiff used the reference transcripts as a gene transfer format (GTF) file and the three sequence alignment map (SAM) or binary version of SAM (BAM) files containing the fragment alignments of the three biological replicates of each sample of the three small intestine regions. To analyse whether genes were ubiquitously expressed among the targeted tissues, a venn diagram was plotted by using Venny, a freely accessible (http://bioinfogp.cnb.csic.es/tools/venny/index. Html) interactive tool for the comparison of lists. The multidimensional scaling plots (MDS), which measure the similarity of the samples and project it into two dimensions, were performed to measure the relationship between duodenum, jejunum and ileum using cummeRbund package in the R statistical environment (Trapnell et al., 2012). Principal component analysis (PCA) was also used for clustering and exploring the relationships between conditions. Graphs were generated by using cummeRbund and the ggplot2 package (Wickham, 2009). Visualisation of results was also undertaken using the cummeRbund package in R (Trapnell et al., 2012).

2.8. Functional annotation and pathway analysis

For each DEG, Gene Ontology (GO) annotation was done by using blast2go (Liu et al., 2011), which uses a blast algorithm to assign GO terms to sequences based on similarity. This was done to identify the key pathways and interactions that might be relevant for the response of intestinal tissues to *A. galli* infection and to annotate significant interactions with metabolic pathways. The DEGs between infected and non-infected intestinal tissues were classified into the categories of biological process, molecular function and cellular components. Bar graphs illustrating similarities and differences between GO terms were plotted using Web Gene Ontology Annotation Plot (WEGO), a web-based tool (Ye et al., 2006).

2.9. Validation of RNA-seq results with RT-qPCR

Real time quantitative PCR (RT-qPCR) was performed using QuantiTect SYBR Green kit (Qiagen Inc., Valencia, CA) to test the validity of specific gene transcription, RNA-seq data and variations in gene expression among individuals. The RNA used for qRT-PCR was prepared in the same way as the total RNA extraction and DNase I treatment described above. Five genes were selected based on their functions in the immune response as reported in other chicken studies and significance within the RNA-seq results (Pasick et al., 2017; Smith et al., 2015; Rychlik et al., 2014). Primer pairs were designed using Primer3 (Rozen and Skaletsky, 2000) and their primer sequences are listed in Table 1. A reference gene (*β*-actin) was used as endogenous control for normalization of the real-time PCR analysis. Reverse transcription (RT) of RNA was achieved using iScript Advanced first strand cDNA synthesis kit (Bio-Rad Laboratories Inc., Hercules, CA, USA) in a total reaction volume of 20 μl following the manufacturer’s instructions. Quantitative PCR was performed in triplicate for each cDNA sample on Roche LightCycler® 96 System (Roche Applied Science, US) using KAPA SYBR FAST Universal qPCR kit (KAPA Biosystems, SA) according to the manufacturer’s instructions. Threshold lines were adjusted to intersect amplification lines in the linear portion of the amplification curve and cycles to threshold (Ct) were recorded. Standard curves for each gene and the reference gene were constructed using serial dilutions. The relative gene expression level was estimated using the comparative cycle threshold (CT) method (Livak and Schmittgen, 2001; Pfaffl, 2004). The method calculates the ratio of relative expression from the efficiency (E) and threshold cycle (Ct) values of an unknown sample versus a control sample.

### Table 1

| Primer name | Forward | Reverse |
|-------------|---------|---------|
| FABP1       | GGGAGAGGTGAGATGAGA | CCGCTTCAAGTTAGCAACCA |
| APOA4       | GACCTAACCACACGGCCTAATT | TTCTGAGCTCTTCAAGTGCTCTTC |
| DGAT1       | TGAGCTCATGACGATTCTCTTT | GATCTGCTGCTGCTGCTG |
| BLB2        | CCGGAGGTGTCTGCTTCTC | CCGGAGGTGTCTGCTTCTC |
| MR1         | CACGTGTGTTGAGTTTGGG | CATCTCAGAGGAATATG |
| *β*-Actin*  | AGTCTCTAGCTGATTGAGTCAGAG | TGCACTCTGTCAGCAATGCCC |

Gene used as reference is marked *, Primers were designed using Primer3 program.
jejenum and ileum sections respectively. In tissues from KZN, 33, 15 and 18 DEGs were identified in the duodenum, jejenum and ileum sections of the intestine respectively (Table 4).

A total of 31 genes were co-expressed in all the sections of the small intestines collected from Limpopo and 10 genes where common in the three sections collected from KZN. Three (3) genes were differentially expressed in all the analysed samples (Fig. 3). Only 16 genes were exclusively expressed in duodenum while 47 and 25 genes could be detected only in the jejenum and ileum of chickens from Limpopo. In chickens from KZN, 15 genes were exclusively expressed in the duodenum while 50 and 137 genes could be detected only in the jejenum and ileum respectively (Fig. 3). Some annotated genes showed similar

![Fig. 1. Photomicrograph of a section of a normal (A) and A. galli infested (B) Small intestine. Small intestine infested by A. galli worms (C). Necrosis of the crypt of Lieberkuhn gland and villi were observed in infected small intestine.](image)

![Fig. 2. Overlapping differentially expressed genes (up or down-regulated). Common genes were observed in comparisons between infected and non-infected small intestines of chickens from KwaZulu-Natal and Limpopo provinces.](image)
patterns of expression between different sections of the intestine, which included c-c motif chemokine ligand 21 that was down-regulated in duodenum and ileum of positive chickens from Limpopo. Furthermore, a similar pattern of expression was also observed with tyrosine-protein kinase jack3.

### 3.3. Multidimensional scaling plots

Investigation of the MDS plot produced an apparent structure within the data that is consistent with tissue segments. The plot revealed tissue dissimilarity of gene expression patterns between tissues by clearly separating duodenum, jejunum and ileum segments. This was observed with the MDS plot, a PCA plot based on principal components 1 and 2 resulted in a distinct separation based on tissue segments (Fig. 4a[II] and 4b[II]).

#### 3.4. Gene ontology (GO) analyses

In general, fewer significantly enriched GO terms were found in the KZN-N/P (Additional file 1) compared to the Limpopo-N/P (Additional file 2) comparison. GO terms at level 2 were used as representatives (Fig. 5). Of these GO terms, biological processes such as immune response (GO:0006955), response to stimulus (GO:0050896), immune system process (GO:0002376) and signalling (GO:0023052) were enriched. Binding GO such as dopamine receptor activity (GO:0001591)

### Table 2

Differential gene expression between Limpopo village chicken intestines that were negative and positive for *A. galli* parasites.

| Gene Name               | Seq. Description                        | RPKM_N | RPKM_P | log2(fold_change) | p_value |
|-------------------------|----------------------------------------|--------|--------|-------------------|---------|
| Limpopo up-regulated    |                                        |        |        |                   |         |
| inosentence-associated  | protein                                | 65.8579| 2.14685| -4.72707          | 0.005-05|
| TLR4                    |                                        | 420.328| 16.2807| -4.69028          | 0.002   |
| CXC4                    | c-c chemokine receptor type partial     | 395.386| 2.67279| -3.7349           | 0.005-05|
| HSP7                    | heat shock protein beta-7              | 28.3597| 1.14111| -2.95113          | 0.0001  |
| Limpopo down regulated  |                                        |        |        |                   |         |
| LOC419333               | formin-1, partial                      | 56.8579| 2.14685| -4.72707          | 0.005-05|
| LOC100857191           | c-c motif chemokine 21                 | 48.9869| 3.73815| -3.71208          | 0.0005  |
| CXC4                    | c-c chemokine receptor type partial     | 395.386| 2.67279| -3.7349           | 0.005-05|
| HSP105                  | heat shock protein 105 kda isofrom x2   | 26.7937| 2.29904| -3.54279          | 0.005-05|
| SLAMF1                  | signalling lymphocytic activation chain| 88.0741| 16.2089| -2.95113          | 0.0001  |
| t-cell surface glycoprotein | cd3 epsilon chain                  | 94.6376| 20.5752| -2.20153          | 0.0015  |

P- value of < 0.05 is significant.

### Table 3

Differential gene expression between KZN village chicken intestines that were negative and positive for *A. galli* parasites.

| Gene Name               | Seq. Description                        | RPKM_N | RPKM_P | log2(fold_change) | p_value |
|-------------------------|----------------------------------------|--------|--------|-------------------|---------|
| KZN up-regulated        |                                        |        |        |                   |         |
| ATF7IP                  | activating transcription factor 7-interacting protein 1 isofrom x1 | 3.2018 | 179.746| 5.81093           | 0.005-05|
| LOC419333               | c-c motif chemokine 2110               | 98.7552| 4324.89| 5.45266           | 0.0002  |
| CXC4                    | c-c chemokine receptor type partial     | 395.386| 2.67279| -3.7349           | 0.005-05|
| HSP7                    | heat shock protein beta-7              | 28.3597| 1.14111| -2.95113          | 0.0001  |
| KZN Down-regulated      |                                        |        |        |                   |         |
| APOA                    | bifunctional atp-dependent dihydroxyacetone kinase fad-amp lyase | 218.25 | 82.2961| -4.7062           | 0.005-05|
| DPEP1                   | dipeptidase 1                          | 71.1901| 17.6539| -3.28352          | 0.005-05|
| HSP70                   | Heat shock protein 70                  | 76.8445| 9.57549| -3.99273          | 0.00015 |

P- value of < 0.05 is significant.
and ATP binding (GO:0005524) along with cellular processes such as cellular response to stimulus (GO:0051716) and cellular response to stress (GO:0033554) had the highest percentage of transcripts involved. The most predominant biological functions were common amongst the different anatomical segments of the small intestine. The first five predominant biological processes observed for tissue samples from Limpopo provinces were cellular process, biological regulation, metabolic processes, pigmentation and response to stimulus. The GO profiles were similar between specimens from Limpopo and KZN provinces with the exception of biological regulation that was not in the top three of the biological process term in KZN. Immune response terms that included immune system process, response to stimuli and cell killing were also observed in the presence of *A. galli* infection.

### Table 4
Gene expression statistics for comparisons between negative and *A. galli* positive sections of the small intestine of village chickens from KZN and Limpopo provinces.

| Province | Duodenum | Jejunum | Ileum | Duodenum | Jejunum | Ileum |
|----------|----------|---------|-------|----------|---------|-------|
| **Significantly up & down regulated genes** | | | | | | |
| | 76 | 99 | 78 | 33 | 102 | 158 |
| **Significantly up-regulated genes p < 0.05** | 37 | 58 | 34 | 15 | 41 | 16 |
| **Significantly down-regulated genes p < 0.05** | 39 | 41 | 44 | 18 | 61 | 142 |
| **Non-significant genes p > 0.05** | 34976 | 34953 | 34974 | 35019 | 34950 | 34894 |
| **Number of Sequences** | 123 | 64 | 78 | 32 | 79 | 123 |
| **Sequences with blast hits e-value ≤ 1E−10** | 91 | 41 | 40 | 20 | 51 | 91 |
| **Sequences without blast hits** | 32 | 23 | 38 | 12 | 28 | 32 |

Fig. 3. Overlapping differentially expressed genes (up or down-regulated). Common genes were observed along sections of the small intestines of chickens from KwaZulu-Natal and Limpopo provinces.

3.5. Biological pathways associated with chicken’s response to *A. galli* infections

To gain an understanding into pathways involved in the village chicken’s reaction to *A. galli* infection, gene annotation was conducted using enzyme code and enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) database. The top 5 KEGG pathways associated with the most abundant differentially up-regulated (Fig. 6) genes in infected and non-infected chickens from Limpopo included T cell receptor signalling pathway, Drug metabolism - cytochrome P450, Metabolism of xenobiotics by cytochrome P450, histidine metabolism and arachidonic acid metabolism. The impacted T cell receptor signalling pathway involved tyrosine-protein kinase btk, tyrosine-protein kinase csk and tyrosine-protein kinase jak3 genes that play a major role in the immune response. In KZN chickens, pathways impacted included arachidonic acid (Fig. 7), methane metabolism, glycerolipid metabolism, purine metabolism, glutathione metabolism and cyanoamino acid metabolism. Arachidonic acid and purine metabolism were observed to have been impacted in tissues from both provinces. However, genes involved in these pathways were different for the two provinces.

Pathway analysis was also undertaken using all the up and down regulated genes from the three sections of the small intestine (Fig. 6).
Genes up-regulated in the ileum of KZN chickens impacted the most pathways. In Limpopo, the jejunum had the most impacted pathways. Other pathways were impacted in only one or two sections of the intestine. In Limpopo, T cell receptor pathway was impacted in the duodenum and ileum and not the jejunum. Most of the pathways had only one gene involved except for purine metabolism that had four genes involved, followed by Tryptophan metabolism and Pyrimidine metabolism with two genes each. The Linoleic acid metabolism, Arachidonic acid metabolism, Metabolism of xenobiotics by cytochrome P450, Drug metabolism - cytochrome P450 and Purine metabolism pathways were commonly impacted in both provinces. The common pathways were however not impacted in the same sections of the intestine.

3.6. Validation of RNA-seq results using RT-qPCR

RT-qPCR was performed to validate the RNA-seq results with reference to β-actin control gene. Genes were selected based on their functions in immune response and significance within the RNA-seq findings. Of all the five genes tested, MR1 gene was not differentially expressed in the RT-qPCR results (Table 5). However, the other genes were consistent between RNA-seq and RT-qPCR results.

4. Discussion

Nematodes of the genus Ascaridia are known to infect many species of birds causing fatal diseases. The small intestine is a principal target organ of A. galli infection (Katakam et al., 2010; Luna-Olivares et al., 2012; Taylor et al., 2007). Previous study (Fatihu et al., 1990; Luna-Olivares et al., 2012) showed that most of the helminth parasites were restricted to the small intestine, particularly the duodenum where there are optimum concentrations of saline and glucose. The histopathology of the three anatomical sections of the infected small intestines of chickens from Limpopo and KZN showed signs of damage. The histology findings imply different roles of the different sections in the life cycle of A. galli similar to reports from previous studies (Idi, 2004; Kaufmann, 1996; Soulsby, 1982; Luna-Olivares et al., 2012). The exact time of infection could not be established as the chickens were naturally infected with the A. galli parasites. However, adult mature worms were collected from the small intestines suggesting that the infection was at
the late-stage.

An average of 3.97 million paired-end reads per sample were generated, providing sufficient sequence coverage for transcriptome profiling (Sultan et al., 2008). An average of 84.82% of these reads were mapped to the reference chicken genome (gallus.galgal4.74). This suggested that the majority of the reads were of high quality and within RNA-Seq quality standards (Mortazavi et al., 2008) for downstream analysis.

The study reported altered transcriptomes as evidenced by DEGs between the A. galli infected and non-infected intestines. The small intestines of chickens from Limpopo and KZN chickens that were infected by A. galli differed in the quantitative composition of the differentially expressed genes implying differences in the genetic mechanisms between the two chicken populations. More genes and pathways were involved in chickens from Limpopo versus those from KZN province.

Complementary to whole organism analysis, this study provided a finer resolution of the molecular processes happening in the different sections of the small intestine. Danos (2013) observed the strong positive correlation between libraries prepared from pooled RNA samples (pooled RNA from 3 biological replicates) and those from libraries prepared separately from biological replicates. As such and due to financial and computational resource constraints, we decided to use the “pooled” RNA approach to reduce the cost of sequencing. The results that were validated with RT-qPCR method revealed that the different sections of the small intestine differed in the quantitative composition of the genes expressed in response to A. galli infection. The duodenum, jejunum and ileum displayed distinct transcriptome characteristics supporting the hypothesis that different regions of the intestine are impacted differently during nematode invasion. It was not surprising that the ileum, which is the lower small intestine, known to be associated with increased immune surveillance had the largest or second largest number of expressed genes in chickens from KZN and Limpopo provinces respectively. In a study by Freeman et al (2012), the largest cluster of genes associated with the immune system were observed to be expressed two- to three-fold higher in the ileum relative to the other regions.

Several genes involved in the immune response were observed to be differentially expressed in this study. The DEG analysis indicated that

Fig. 5. Enriched gene ontology terms. Ontology categories resulting from interactions between chicken and A. galli infection from Limpopo and KZN.
the MHC class II m alpha chain precursor was up regulated whilst MHC class II partial was down regulated in chickens from Limpopo. Class I histocompatibility f10 alpha chain-like isoform x1&2 were also down regulated in Limpopo. The MHC (Major Histocompatibility Complex) is a group of genes present in all vertebrates that plays a major role in immune response mechanisms through molecules known to regulate different cellular components of the immune system including the T cells, B cells, and antigen-presenting cells (Lamont, 1998). The chicken MHC (B complex) has been implicated with conferring either resistance or susceptibility to a variety of viral, parasitic, and bacterial diseases (Lamont, 1998).

Pro-inflammatory cytokines genes were also observed to be differentially expressed in the study population. The expression of pro-inflammatory cytokine genes are known to be activated by toll-like receptors (TLRs) in order to directly control parasite replication (Gazzinelli and Denkers, 2006). Interleukin-20 receptor subunit alpha and...
Table 5

| Comparison                     | Method       | ApOA4 | MR1  | FAB1 | DRD  | BLB2 |
|--------------------------------|--------------|-------|------|------|------|------|
| Duodenum N/P                   | RNA-seq      | 2.85  | 0.57 | 2.41 | 0.46 |      |
|                                | RT-qPCR      | 3.11  |      | 2.04 |      | 1.58 |
| Jejunum N/P                    | RNA-seq      |       |      |      |      |      |
|                                | RT-qPCR      | 2.85  | 0.57 | 2.41 | 0.46 |      |
| Ileum N/P                      | RNA-seq      | 1.58  | 2.61 | 1.43 |      |      |
|                                | RT-qPCR      | 0.78  | 1.79 | 0.73 |      |      |

* N = Negative; P = Positive.

b Represents gene that was not significantly differentially expressed in RT-qPCR results.

Interleukin-21 receptor were up-regulated in Limpopo chickens whereas interleukin- partial was down-regulated in KZN. Interleukin-22 is a member of the interleukin 10 related cytokine family (Zenevitz et al., 2008). The interleukin-22 receptor is highly expressed within tissues, such as epithelial cells of the gastrointestinal tract.

Different families of pattern recognition receptors (PRRs) have been identified (Mair et al., 2004) including among other receptors, TLRs (O’Neill, 2006). TLRs play a central role in the initiation of immune responses against a number of pathogens (Ingham et al., 2008). They recognize various pathogen-associated molecular patterns (PAMPs) from parasites (Kawai and Akira, 2001). TLR7 is commonly involved in pathogen recognition, and was up-regulated (4.2 fold increase) in infected chickens from Limpopo (Limp5). In a separate study (Ingham et al., 2008), TLR7 transcript level changes were observed in sheep resistant to H. contortus. Previous studies on the identification of avian TLRs were primarily dependent on bioinformatics (in silico) approaches (Temperley et al., 2008; Yilmaz et al., 2005). Limited information is therefore available for avian TLRs’ functions and mechanisms of regulation. This study provides unique information important in understanding the involvement of avian TLRs in the immune response to nematode infections.

Cytochrome P450s (CYPs) are a large superfamily of enzymes found in almost all living organisms. In this study, Cytochrome P450 2h1-like isofrom x1 was down regulated in infected KZN chickens and not differentially expressed in the chickens from Limpopo region. In a study on malabsorption syndrome, which is a model for intestinal disturbances in young chickens (van Hemert et al., 2004), Cytochrome P450 and apolipoprotein B showed differences in expression among lines. Aplipoprotein A-iv was down regulated in chickens from KZN. Previous studies reported down regulation of apolipoprotein B and cytochrome P450 in intestinal epithelium in response to pro inflammatory cytokines (Besnard et al., 2002; Darnawi et al., 2013). Based on such evidence, the down-regulation of apolipoprotein and cytochrome P450 in this study, might be a response to intrusion in the small intestine.

The CXC chemokine receptor type partial and CXC chemokine 21 were down regulated in the small intestines of A. galli infected chicken from Limpopo province. CXC chemokines IL-8 and K60 were up regulated in the jejunum of Salmonella serovar Typhimurium infected chicken (Withanage et al., 2004). A Th2 polarised cytokine response was reported in the jejunum and spleen of A. galli-infected chickens (Kaiser, 2007; Pleidrup et al., 2014). An increased expression of the Th2 signature cytokine IL-13 in the spleen of A. galli-infected chickens was also observed in this study. CXC activates natural killer cells and is thought to play a role in the temporal development of innate and adaptive immunity (Neville et al., 1997). As suggested by results from this study, CXC seem to have a major role in the defense of chickens from A. galli. This gene family also was also proven to trigger necrosis (Gasser and Newton, 2000), which is consistent with the histology results from this study.

Heat shock protein 30c-like and heat shock protein beta-7 were up regulated while heat shock protein 105 partial was down regulated in Limpopo chickens. Heat shock protein 30c-like belongs to the small heat shock protein 20 family and is involved in stress response. Heat shock protein 70, which can also play a significant role in heat stress, underwent significant down-regulation in infected chicken from KZN. This stress protein was also expressed in other studies (Kim et al., 2014).

Notably, this study revealed several genes that were specific to different segments of the intestine and are important in immune response mechanisms. MDS plots highlighted the separation of the three targeted sections of the intestine into three distinct groups, results of which were consistent with the PCA analysis. Taken together, both the MDS and PCA suggest significant biological dissimilarities between segments, which may be a result of the tissue’s degree of specialization. Results from this study are consistent with reports by Mach et al (2014) who investigated gene expression patterns along the proximal-distal axis of the porcine small intestine (duodenum, jejunum and ileum) and observed an obvious structure along tissue segments.

The analysis of enriched GO terms enabled the discovery of significant gene categories that could have been overlooked by evaluating individual genes. The enriched GO terms could aid in interpreting the dominant functions controlled by DEGs. The higher number of identified GO terms was positively correlated to the number of DEGs in all comparisons. This trend was observed in both up and down regulated genes. The enriched biological processes such as response to stimulus, immune system process and signalling are all involved in immune response and could be associated with the resistance or tolerance of chickens to A. galli parasites.

The biological pathway analysis gave insight into the molecular mechanisms underlying protective immunity and host resistance to A. galli parasitic infection. Arachidonic acid metabolism was one of the pathways significantly impacted in A. galli infected chickens from KZN. Arachidonic acid is capable of modifying or regulating one or more immune functions and may serve as a potent inhibitor for Type 1 helper T cell (Th1) response (Freeman et al., 2012). It is one of the crucial polyunsaturated fatty acids associated with membrane phospholipids which when liberated from the plasma membrane, can be oxidized to a variety of eicosanoids. Eicosanoids act in signalling molecules and stimulating a variety of responses in their target cells including an immune response (Peters-Golden et al., 2005). Worm killing activities of arachidonic acid have previously been demonstrated in a study conducted in mice where a single oral dose of arachidonic acid led to a significant reduction of total worm burden of Schistosoma.

Another pathway mostly impacted was the T cell receptor signalling pathway. The ability of Nematodes to destabilize the TLR signalling pathway has been reported previously (Kane et al., 2004). Our results suggest a role of T-cell receptor signalling pathway in invoking effective host immune responses and in the development of host resistance.

5. Conclusion

The study used RNA-seq data to successfully identify and analyse DEGs from small intestine collected from village chickens that were naturally infected with A. galli. There were noteworthy differences in the mechanisms of host resistant to A. galli infestation between chickens from different provinces, which could be due to variance in genetic profiles of the two populations. The data presented in the study could find application in development of genomic markers for use in selection and breeding of chickens against natural infections with pathogens such as A. galli. Understanding the molecular mechanisms that contribute to protective immunity, immune suppression, pathology, and host resistance will have a significant impact on alternative disease/Pathogen control strategies.

Availability of data and materials

The datasets supporting the conclusions of this article are available
in the NCBI Short Read Archive (SRA) repository, SRP070940.txt.

Competing interests

There authors declare that they have no competing interests.

Authors’ contributions

DPM performed lab work, analysed data and drafted the manuscript. FCM was the project leader and engaged in the structure of the manuscript. EvM participated in the coordination and preparation of the manuscript. All authors were responsible for experimental and project design.

Ethical standards

Ethical approval for the study was obtained from the Animal Ethics Committee from the Agricultural Research Council-Animal Production Institute (APIE1C/004) and University of Pretoria (EC090-13).

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