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Identification and Validation of Differentially Expressed Genes Via-s-vis Exploration of the Modular Pathways in Diseased Versus Healthy Nili Ravi Water Buffalo

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Abstract

Peripheral blood mononuclear cells (PBMCs) were isolated from 3 groups of she-buffaloes (Tuberculosis, Metritis, and Healthy control) was sequenced by RNA-Seq (using Illumina Hiseq 2500 platform). The pre-processed reads, obtained from transcriptome sequencing, were aligned to the Bostaurus genome using the Hisat-2 program. Gene expression was studied using the String Tie program. A total of 31982 transcripts were identified. Comparisons of the entire 3 groups' revealed 176 differentially expressed genes (DEGs) in TB vs. healthy groups and 162 DEGs in metritis vs. healthy groups. Analysis of gene ontology and pathways (molecular function and biological processes) identified certain pathways like cytokine activity, Wnt signaling, PI3K-Akt signaling, MAPK signalling (between TB and healthy groups) and cAMP signaling, Wnt signaling, TGF-beta signaling, MAPK signaling, PI3K-Akt signaling, etc. between metritis-positive and healthy buffaloes. Network analysis identified the immune-related genes contributing to the system biology related to the disease-resistance in Nili Ravi buffalo. Besides, five differentially expressed genes have been validated using SYBR-green chemistry of qPCR. In the future, these key genes could be studied in detail to explore their potential to be promising biomarkers for selecting breeding animals with higher tolerance against these economically devastating diseases.

Keywords: Transcriptome sequencing • RNA-seq • qPCR • Differentially expressed genes • Pathway analysis • Disease-resistance

Introduction

Buffalo has been an integral part of livestock farming and agriculture in Asia for over 5000 years and played a pivotal role in overall agro-socio-economic development through contributing meat, milk, hide, and draft power. India treasures about 57.3% of the world buffalo population (which is 109.85 million as per the 2019 national livestock census of India) [1]. Buffalo contributes 55% of the total milk production (165.4 million tonnes) in India [2]. Nili Ravi, an important bubaline breed in its natural breeding tract Punjab (India and Pakistan), is considered as a producer of milk with a high-fat content (7-8.5%), meat, and remarkable ability to utilize poor quality fodder.

Scientific knowledge on the incidence of clinical diseases in dairy animals allows identifying factors and assists in formulating the priorities of breeding and disease-prevention programs [3]. Genetic predisposition plays an important role in developing resistance to infectious diseases like brucellosis, hemorrhagic septicaemia (HS), tuberculosis (TB), mastitis, rinderpest, Johne's disease (JD), etc. Besides, some of these diseases have a zoonotic impact on public health. A study on the occurrence of TB in cattle and humans (in India) reported that 15.7% of humans had *Mycobacterium tuberculosis*, 26.8% of cattle had *M. bovis*. However, 8.7% of humans had *M. bovis* TB and 35.7% of cattle had 'mixed infections [4]. TB has no geographical boundaries and infection occurs in a diverse group of animals [5]. Similarly, postpartum metritis is one of the most important disorders in bovines, causing high economic losses due to prolonged days-open as well as inter-calving periods, leading to involuntary culling. The incidence rate of uterine infection in she-buffalo (24.7) is found to be much higher than in cows [6].

Global transcriptome analysis helps unravel all the transcripts available in a cell type or tissue. The massive parallel sequencing approach enables us to determine the differential expression profile of the genes between the experimental groups and to identify the significantly associated modular genes [7-9]. The detailed systems biology and related genes associated with metritis and tuberculosis are not known in buffalo [10,11]. Research endeavors directed towards discovering the underlying genes contributing to susceptibility/tolerance of Nili Ravi buffaloes to infectious diseases have been done been undertaken. Increasing evidence suggests that mRNA expression plays an important role in host-pathogen interactions through modulation of both innate and acquired immune responses [12,13]. The gene and miRNA expression profile in the PBMCs alters due to diseases [14] like TB and metritis. Considering the gap in knowledge on systems biology associated with disease resistance in Nili Ravi buffaloes, the present study was designed to discover the mRNA repertoire and explore the modular genes and pathways associated with resistance/susceptibility to tuberculosis (TB) and metritis in this economically important breed of water buffalo.

Materials and Methods

Sample collection and experimental design

Six adult female Nili Ravi water buffaloes (aged between 2 to 4 years),

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maintained under similar management in the organized institutional dairy farm of Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana, India were included in the study. These animals were divided into 3 groups (two biological replicates each): Tuberculosis positive (TB), Metritis positive (Met), and Healthy control (Ctrl) animals. Peripheral blood samples (10 ml) were aseptically collected from the jugular vein in tubes containing 500 µl of 0.5M EDTA and immediately brought to the laboratory to process for PBMCs isolation by density gradient centrifugation method [15,16]. We have used PBMC for comparison of RNA expression because the diseases have manifestations in the PBMCs due to their involvement in innate immunity. The gene expression profile in the PBMCs alters due to diseases like TB and metritis [17,18]. This research work was certified by the Institutional Animal Ethics Committee (IAEC). All the experiments and protocols were conducted at the College of Animal Biotechnology, GADVASU.

RNA extraction

The total RNA from all 6 samples was isolated using the mirVanaRNA isolation kit (Ambion, Life Technologies, USA). The RNA samples with OD (260/280) between 2.0 and 2.2 (assessed by Nanodrop, Thermo Fisher) were selected for further processing.

Transcriptomic sequencing

The total RNA samples were outsourced to Agri-Genome Labs Private Limited, Kochi, Kerala, India by maintaining proper cold-chain (using dry ice) during transit, for next-generation sequencing (NGS) using Illumina Hiseq 2500 platform (100 bp paired-end reads) and preliminary biocomputational analysis of the raw data.

Analysis of sequenced data

The raw data were pre-processed using the FASTX tool. About ~94% of the total reads of all the samples passed ≥ 30 Phred score (Table 1). The quality of the reads was checked for each of the samples using sequence quality score distribution, average base composition, average base quality, GC% distribution, check for over-represented sequences as well as biasing of kmers, and read-length distribution of forward and reverse reads (Table 2). The raw sequence reads in FASTQ format have been published through NCBI SRA (https://www.ncbi.nlm.nih.gov/sra/PRJNA514883) with the experiment accession numbers SRX5282354 to SRX5282359.

The quality passed reads were subjected to adapter trimming and then the pre-processed reads were aligned to the taurine genome (UMD3.1) [19] downloaded from Ensembl database (ftp://ftp.ensembl.org/pub/release-89/gtf/bos_taurus/Bos_taurus.UMD3.1.89.gtf.gz) using Hisat2 program (version 2.0.5) with default parameters. Then the aligned reads were used for estimating expression of the genes and transcripts, using the String_Tie program (version 1.3.3b).

Identification of differentially expressed genes (DEGs)

The average FPKM values of two biological replicates belonging to each of the experimental groups were calculated. The non-coding RNA genes (viz. miRNA, snoRNA, and snRNA) were removed from the list of 31982 genes for each of the experimental groups. The differentially expressed coding mRNA genes were identified between the healthy groups with each of the diseased groups. The common and unique protein-coding genes among the experimental groups being compared and were represented by the Venn diagram using online web-tool IneractiVenn (http://www.interactivenn.net/) and R Program (v.3.2.0) package Venn Diagram v.1.6.9s. The fold change of expression was calculated by taking the logarithm (with base 2) of the ratio of expression values between the two groups (healthy versus diseased) being compared. The genes with fold change were considered as differentially expressed genes. Expression heatmaps (on the color scale bar; white-orange-red; representing the low-medium-high expression, respectively) of the mRNAs for the experimental samples were constructed using the WGCNA package [20] of R program (v.3.2.0).

Validation of selected DEGs using qPCR

Five differentially expressed genes were selected for validation of the obtained transcriptomic analysis results (primer details given in Table 3). The expression of these genes in the whole blood or leukocytes was checked from the Genecards database (www. genecards.org/). The primers were designed using the available mRNA sequences in NCBI Nucleotide (https://www.ncbi.nlm.nih.gov/nuccore/) using primer-BLAST (https://www.ncbi.nlm.nih.gov/ tools/primer-blast/) online tool. The specificity and sensitivity of the designed primers were checked by IDT Oligo Analyzer (v.3.1.1) (https://eu.idtdna.com/pages). Expression of selected differentially expressed genes was quantified (in three technical replicates) by real-timePCR (Bio-Rad) using a miScript SYBR-Green PCR kit (Qiagen, Germany) and beta-actin as an endogenous control.

Total RNA was isolated (for validation using qPCR) from PBMCs extracted from fresh blood samples (three biological replicates of each of the 3 experimental groups) using PureLink® RNA Minikit (Ambion) and then reverse-transcribed into cDNAs bymiScript II RT Kit (Qiagen, Germany)

Table 1. Quality scores of the 6 RNA samples subjected to transcriptomic sequencing.

Sample Name*	#of raw reads (paired-end)	#of bases (Gb)	GC%	%of data ≥ Q30	Raw read length (bp)
TB_1	27, 074, 735	5.4	51.11	93.8	100 × 2
TB_2	27, 233, 730	5.4	51.44	94	100 × 2
Metritis_1	25, 307, 169	5	49.41	92.8	100 × 2
Metritis_2	26, 758, 839	5.3	52.56	95	100 × 2
Healthy_1	26, 647, 077	5.3	52.29	95.5	100 × 2
Healthy_2	27, 316, 174	5.4	52.66	95.4	100 × 2

*TB_1: Tuberculosis Positive Sample 1; TB_2: Tuberculosis Positive Sample 2; Metritis_1: Metritis-Positive Sample 1; Metritis_2: Metritis-Positive Sample 2; Healthy_1: Healthy Control Sample 1 And Healthy_2: Healthy-Control Sample 2

Table 2. Quality checking and detail of read-counts of the 6 experimental samples subjected to paired-end sequencing using Illumina Hiseq 2500 platform.

Sample Name*	Total Reads	QC Passed	QC Passed%	Aligned read-count	Aligned %	Unaligned Read-count	Unaligned %
TB_1	54149470	54114736	99.93	37084829	68.53%	17029907	31.47
TB_2	54467460	54417426	99.9	37368446	68.67%	17048980	31.33
Metritis_1	50614338	50574744	99.92	32590365	64.44%	17984379	35.56
Metritis_2	53517678	53419886	99.81	36688778	68.68%	16731108	31.32
Healthy_1	53294154	53238892	99.89	36191799	67.98%	17047093	32.02
Healthy_2	54632348	54600404	99.94	37434037	68.56%	17166367	31.44

*TB: Tuberculosis Positive Sample 1; Metritis: Metritis-Positive Sample; Healthy: Healthy Control Sample

Table 3. Detail of the primers used for validation of expression profile of the selected differentially expressed genes by real-time PCR (SYBR Green chemistry).

Primer name	Sequence (5'-3')	Length	NCBI Accession Number	Tm (ºC)	GC%
Genes for validation: Tuberculosis-positive vs. Healthy control groups					
TOMM7-F	ctcagtgctcctctcctttctg	22	XM_006074849.1	60.09	54.55
TOMM7-R	ttcagggtacagatgaggcaac	22		60.03	50
SPTSSB-F	agctacggagatatttgggctg	22	XM_006062959.1	59.96	50
SPTSSB-R	cgctcaattatcttccccagga	22		59.9	50
MSH4-F	cgaaaactttggccagctgatt	22	XM_006060238.1	60.29	45.45
MSH4-R	tctccctacctttcacctctgt	22		59.89	50
Genes for validation: Metritis-positive vs. Healthy control groups					
MRPL33-F	aagtagttgtttgttgctggcc	22	XM_006046139.1	59.9	45.45
MRPL33-R	cagtttctcccatagtcggctt	22		60.09	50
GPC3-F	ttatccagccgaagaagggaac	22	XM_006074811.1	60.09	50
GPC3-R	ttccattccttgctgccttttg	22		59.96	45.45
Endogenous control beta-actin					
ACTB-F	atgatattgccgcgctcgtg	20	NM_001290932.1	61.56	55
ACTB-R	catcccccacgtacgagtcc	20		62.01	65

according to manufacturer's protocols. For each sample, a 20 µl reaction was setup on the ice having 4 µl of 5x miScriptHiSpec Buffer, 2 µl of 10x miScriptNucleics Mix, 2 µl miScriptTranscriptase Mix, 5 µg of RNA sample and RNase free water to make up the volume to 20 µl. Reaction tubes were incubated for 37ºC for 60 mins, 95ºC for 5 mins in a Veriti thermal cycler (Applied Biosystems).

Real-time PCR was conducted in triplicate (3 technical replicates) using miScript® SYBR® Green PCR Kit (Qiagen) using BioRAD CFX96Real-time PCR System as per manufacturer's protocol (i.e., cycling conditions of 95ºC for 15 min, followed by a total of 40 cycles of 94ºC for 15 sec and 55ºC for 30 sec and 70ºC for 30 sec). The reaction mixture was prepared using 10 µl of 2x Quanti Tect SYBR Green PCR Master Mix, 2 µl of 10x miScript Universal Primer, 2 µl of each forward and reverse primer, 2 µl of template cDNA and nuclease-free water to make the final volume 20 µl. For each assay, a no-template control (NTC) and No RT control reaction were also included.

Functional annotation and pathway analysis

The differentially expressed protein-coding genes obtained by comparing the diseased and the healthy groups were analyzed for gene ontology and then subjected to pathway analyses using Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8 (https://david.ncifcrf.gov/) [21,22] and Panther Classification System v11.1 (http://www.pantherdb.org/loginRequired.jsp?access=true) [23,24]. The pathway analysis results (with enrichment score 1.0 and medium stringency) deciphering the biological functions of the DEGs in systems biology have been presented as pie-chart. The gene-groups below 1.0 enrichment scores have not been considered for further analysis. The important pathways identified from DAVID (based on the available literature, as discussed in the Result and discussion section) were selected for identifying the important genes. The Ensembl Gene Ids were converted to Protein Ids using g:Convert Gene ID Converter of g:Profiler (https://biit.cs.ut.ee/gprofiler/index.cgi) [25]. The proteins encoded by the selected DEGs for each pair of groups being compared were then analyzed through String DB [26] to construct the protein-protein interaction (PPI) network.

Results and Discussion

Uniquely and differentially expressed genes

A total of 31982 transcripts (Figure 1A) were identified which included 15772 protein-coding mRNAs (inclusive of multi-copy of some genes) and 16210 non-coding RNAs (miRNA, misc_RNA, Mt_rRNA, Mt_tRNA, rRNA, snoRNA, and snRNA), processed-pseudogenes, pseudogenes, and other unidentified transcripts. The Venn diagram (Figure 1B) depicts that 8132 mRNAs were commonly detected in all three groups. The Fragments per Kilobase of transcript per Million mapped reads (FPKM) values of each of the six samples for all the 31982 genes have been tabulated in Supplementary Table S1. While comparative analysis between the diseased and healthy groups revealed uniquely expressed mRNAs that are likely to be associated with disease resistance or susceptibility viz., 411 mRNAs specific to Tuberculosis-positive group and 468 mRNAs to the healthy control group, 373 and 469 uniquely expressed mRNAs in Metritis-positive group and healthy control group, respectively. The number of unique genes of TB, metritis, and healthy groups was 231, 193, and 273, respectively.

Identification of differentially expressed genes

The differentially expressed protein-coding genes were identified as up and down-regulated based on log2 (FPKM) >2 and <-2, respectively. The detailed annotations of all 31982 genes, as well as the lists of all the differentially expressed vis-à-vis, uniquely expressed protein-coding genes are available in the Supplementary Tables S2 (TB vs. Healthy comparison) and S3 (Metritis *vs*. Healthy comparison). In total, 162 differentially expressed (up- and down-regulated) mRNA genes (DEGs) were identified in 176 DEGs (mRNA) in TB *vs*. healthy group (Supplementary Table S2a) and metritis *vs*. healthy group (Supplementary Table S3a). The expression profile of the protein-coding genes has been represented in the heat map (Figure 2). It indicated that treatment group 2 (i.e. metritis samples) along with a healthy control group exhibited the most similar mRNA expression profiles, while TB group exhibit a distinguished mRNA expression pattern.

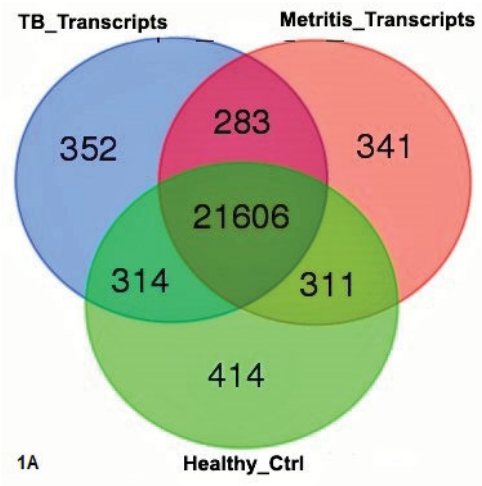

Figure 1A. Venn diagrams representing common and unique transcripts (inclusive of mRNAs and other non-coding RNAs) across the experimental groups.

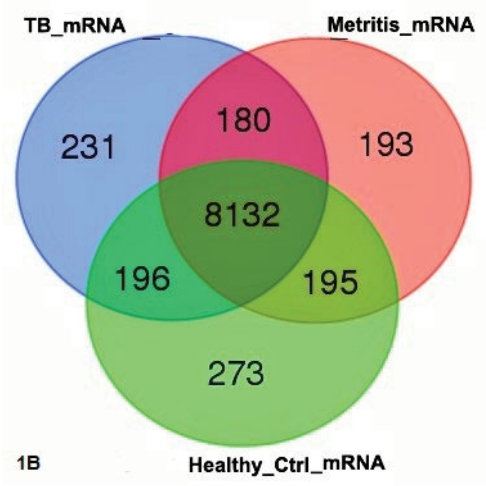

Figure 1B. Venn diagrams representing common and unique mRNAs (protein coding genes only) across the experimental groups.

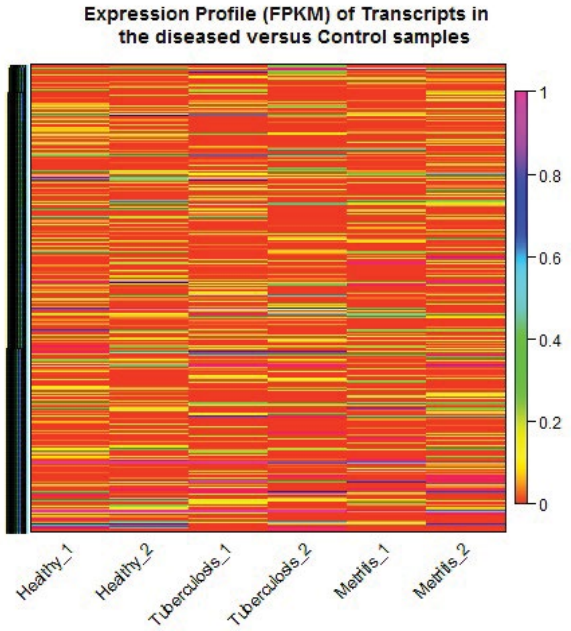

Figure 2. Expression profile (FPKM) of transcripts of the differentially expressed genes in the experimental samples.

Validation of DEGs by qPCR

Real-time PCR was performed for gene expression study of selected DEGs (Figure 3). The selected 5 differentially expressed genes (3 genes for TB *vs*. Healthy and 2 genes for Metritis *vs*. Healthy comparative-pairs) were analyzed for fold-change using the ddCt method [27]. The FPKM values of MSH4 and TOMM7 genes showed up-regulation (mean FPKM values 0.19 and 15.67, respectively) and SPTSSB was found to be down-regulated (mean FPKM value 0.00) in Tuberculosis samples as compared to the healthy ones (0.02, 0.00 and 0.71, respectively, for MSH4, TOMM7, and SPTSSB), as obtained from the Illumina next generations sequencing of the transcriptome (Supplementary Table S1). TB is characterized by high bacterial load and weight loss leading to reduced survival [28]. The results of the qPCR analysis showed that TOMM7 was up-regulated while MSH4 was down-regulated (fold-change 1.21 and 0.83, respectively) in TB as compared to the healthy group and SPTSSB was almost same in both experimental groups. Ontology study and functional analysis of these genes revealed that the Translocase of Outer Mitochondrial Membrane 7 (TOMM7) gene encodes a subunit-peptide of the translocase enzyme, which regulates the assembly and stability of the TOM complex [29]. It is well known that host-mitochondria are snipped-targets of several bacteria including *Mycobacterium* [30,31]. The MutS Homolog 4 (MSH4) gene encodes a meiosis-specific protein that forms a heterodimer with MSH5 to bind to a Holliday Junction and thereby provoke ADP-ATP exchange. Mutation of this protein is associated with altered or mismatched DNA-binding during Meiosis-I thus affecting the cell-cycle. Host-cell death is caused by the host-pathogen interaction to prevent host immunity to overcome the *Mycobacterium*-infection and promoting host cell necrosis [32]. It has been propounded that *Mycobacterium* promotes programmed necrosis of host-cells and thwarts host cell apoptotic signalling [33]. The up-regulation of TOMM7 and down-regulation of the MSH4 gene in the TB-positive samples are logical and also validated in our qPCR study. The Serine Palmitoyltransferase Small Subunit B (SPTSSB) gene encodes a peptide that acts as a small SPT subunit associated with SPT activity and also involved in conferring acyl-CoA preference to the SPT catalytic heterodimer of SPTLC1 [34]. Although this gene was selected based on its expression in the blood cells (so that we can easily detect in the PBMCs) available at the Gene Cards database (www.genecards.org), it seems SPTSSB gene has no direct association with resistance or susceptibility to *Mycobacterium* infection. So the TB-positive sample and the healthy sample didn't show a similar pattern of expression as evident in the NGS data.

The other two genes that were selected for validation of Metritis-positive *vs*. healthy groups were GPC3 (down-regulated in Metritic samples) and MRPL33 (up-regulated in Metritic samples), as presented in the transcriptome sequencing data. The MRPL33 has shown up-regulation in the Metritic sample in the qPCR validation experiment. Mitochondrial Ribosomal Protein L33 (MRPL33) is associated with mitochondrial protein synthesis, viral gene translation in infected cells, and organelle biogenesis [35]. In our validation study, the MRPL33 was found to be more expressed (Fold-change 1.18) in the PBMCs of metritis-positive samples. Perhaps, the bacterial infection has stimulated the expression of this gene. Finally, the GPC3h as show almost the same level of expression in both groups (Fold-change 0.99). This gene encodes Glypican 3 protein that forms the membrane-associated protein core of cell surface heparin sulfate proteoglycans. This protein is associated with apoptosis-induction, modulation of growth in mesodermal tissues, and interactions between IGF2 and its receptor [36]. Thus this gene can also be associated with inflammatory responses in the uterine cells. In a recent study on genome-wide association study for identifying the DEGs expressed in the endometrium of Holstein Cows, Stephen et al. identified GPC3 to be expressed poor-fertile cows (logFC=1.61, P=0.03) [37]. There is a difference in transcriptomics expression profile (revealed by NGS) and qPCR results of the selected 5 genes. This could be attributed to using new blood samples for qPCR. Research reports suggest that these genes are involved in the disease regulation pathways [38].

Gene ontology and functional classification

Gene ontology (GO) for biological processes, molecular functions, cellular component, pathway, and protein class of differentially and uniquely expressed genes in the comparison of diseased vs. healthy sample groups was studied using DAVID, Pather D Band String DB. During a comparison between the tuberculosis-positive and the metritis-positive animals with the healthy group, in total, 1055 and 1004 protein-coding DEGs were identified, respectively, for functional annotations using DAVID. The Ensembl Gene Ids were uploaded and 896 (for TB *vs*. Healthy) and 821 (Metritis vs. Healthy) genes from Bostaurus species were selected for further analysis.

Tuberculosis-positive vs. healthy groups

Each of the enriched GO term with a Bonferroni p-value less than 0.01 enriched gene ontology (GO) terms belonged to the following categories:749 (83.6%), 616 (68.8%) and 651 (72.7%) genes were incorporated into the cellular component (CC), molecular function (MF) and biological process (BP), respectively. Hypergeometric distribution was used to find the significance of enrichment and each enriched GO term (Bonferroni p-value <0.01) was adjusted for multiple testing [39]. The hypergeometric distribution calculates the likelihood that the annotation of GO terms to the section of interacting genes is significantly higher than that of the fraction of all the genes in the taurine genome (http://ctdbase.org/

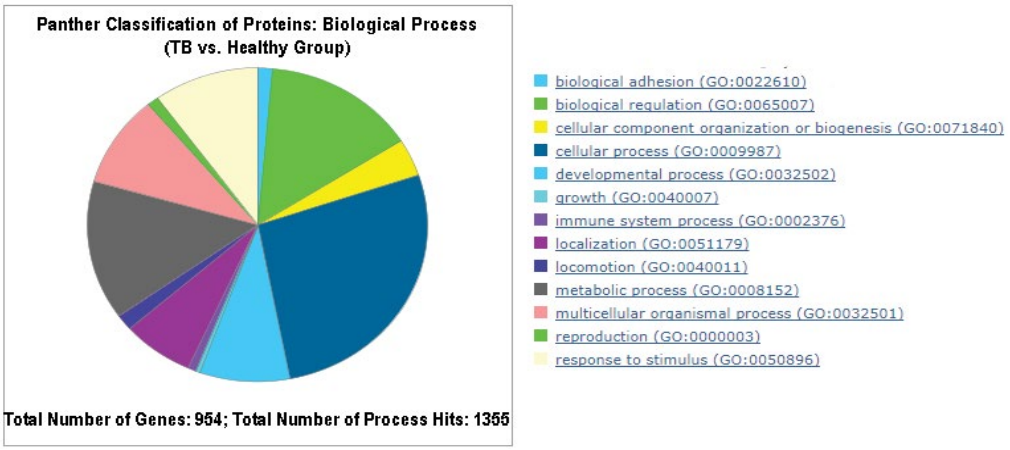

Figure 3. Expression profiling determined by qPCR (SYBR Green chemistry) of the differentially expressed genes selected for comparison in terms of fold change between PBMCs from Diseased vs. Healthy samples.

help/chemGODetailHelp.jsp). The functional annotation chart for the GO terms for molecular function (MF) with the default options (Count=2 and EASE=0.1) identified 36 molecular activities with the lowest p-value=5.0, E-7 and the lowest Bonferroni (2.9, E-4) (Supplementary Table S4). The term cytokine activity (GO:0005125) had 22 gene name records (interleukin 13 (IL13), IL17A, -17F, 2, -21, -22, -36A, -36B, transforming growth factor-beta 2 (TGFB2), GDF-5, 6 and 7 etc.) (Supplementary Table S5). Other important GO-terms enriched for molecular functions are transforming growth factor-beta receptor binding (p=8.34, E-06), growth factor activity (p=4.98, E-04), extracellular matrix structural constituent (p=0.007), cGMP binding (p=0.025), adenylate cyclase activity (p=0.031), cytokine receptor binding (p=0.064) etc. These molecular functions attributed to resistance to bacterial diseases. KEGG pathway analysis of 289 genes with the same threshold as above revealed that the Neuroactive ligand-receptor interaction (bta04080) pathway (27 genes and p=4.50, E-05) was the foremost one. Among the other pathways identified the important ones are Tryptophan metabolism (bta00380) (p=4.79, E-05), TGF-beta signaling (p=6.46, E-05), cAMP signaling (p=5.32, E-04), Wnt signaling (p=0.015), Cytokine-cytokine receptor interaction (p=0.019), PI3K-Akt signaling (p=0.042), MAPK signaling (p=0.063) and GMP-PKG signalling (p=0.082) pathways (Supplementary Table S6). The pathway analysis of tuberculosis susceptibility/resistance reveals that resistance to mycobacterial infection needs TH1 biased cell-mediated immunity [40,41] attributed to several cytokines viz. IL-2, IL-12, TNF, and IFN-γ. By contrast, the anti-inflammatory cytokine IL-10 increases the susceptibility to tuberculosis in humans and mice [42-45]. The common pathways associated with TB infections involve lung-cell necrosis and neutrophil influx [28]. These authors conducted a study on pathways associated with Tuberculosis susceptibility to heterogeneous mice populations. Susceptibility to TB was strongly correlated ($p<0.05$) with neutrophil-infiltration (neutrophilia in a later stage) [46] as well as lung neutrophil chemokines (CXCL1, CXCL2, CXCL5) and tumor necrosis factor (TNF) coupled with cell death. However, the immune cytokines were not strongly associated with TB-susceptibility. They identified that the cytokine 'chemokine (C-X-C motif) ligand 1' (CXCL1) as an important peripheral biomarker of disease. Transcriptomics study on tuberculosis-infected human patients has uncovered a set of dysregulated genes that are components of the PI3–Kinase pathway [47]. Besides, IL-1β, a pro-inflammatory cytokine, modulates the acute inflammatory processes in the central nervous system [48,49]. In our study, functional annotation clustering of the 896 genes with high classification stringency and default threshold parameters revealed 75 clusters with the highest Enrichment Score of 6.85 (Supplementary Table S7).

In total 41 genes were identified contributing to these pathways. The Protein-Ids of these genes were extracted using g: Profiler and then subjected to String DB for generating protein-protein interaction (PPI) network (PPI enrichment p-value <1.0, e-16 and mean local clustering coefficient=0.458). Available literature indicates that these pathways are very critical in susceptibility or resistance to tuberculosis in humans and mice.

Panther analysis of the DEGs (between TB vs. healthy)identified a total of 1355 biological process hits were predicted which was categorized into 13 groups (including cellular process, metabolic process, biological regulation, response to a stimulus, immune system process, etc) (Figure 4).

It is evident from the protein-protein interaction network that insulin-like growth factor 1 (IGF1) forms a strong network (high combined score) with several proteins, like DCN, TEK, SLC24, CSF3, CSF2, MAPK10, IL13, etc. (Supplementary Table S8). Besides, CXCL14, GJA1, DCN, CCL11, CCL25 reveals strong interaction with several genes (Figure 5). The co-expression data shows that many genes with immune-related direct functions (IGF1 with DCN, SLC24, TEK; CSF1 and CSF2, etc.) are co-expressed. Network analysis using String DB identified the immune-related genes (viz, CSF2, CSF3, CXCL14, etc.) contributing to the system biology related to the disease in Nili Ravi buffalo. The gene interaction network of DEGs was studied with a list of combined scores of gene pairs. The combined network enables a topological characterization of the reliability strength of gene associations. A detailed and thorough investigation of the ontology and functional analysis of the 52 genes incorporated in the protein-protein interaction study revealed that these genes are actively involved in chemokine related functions, immunity, binding of molecules, etc. For example, Colony-stimulating factor 2 and 3 (CSF2 and 3: involved in cytokine activity; granulocyte-macrophage colony-stimulating factor receptor binding; growth factor activity), Interleukin-13 (IL-13: cytokine activity; cytokine receptor binding), Placenta growth factor (PGF: growth factor activity; vascular endothelial growth factor receptor binding), Growth/differentiation factor 6 (GDF-6: cytokine activity; growth factor activity);CCL25 protein (CCR10 chemokine receptor binding; chemokine activity); Adenosine receptor A1 (ADORA1: G-protein coupled adenosine receptor activity); C-X-C motif chemokine ligand 14 (CXCL14: chemokine activity; cell-cell signaling; cell chemotaxis; immune response; positive regulation of natural killer cell chemotaxis),etc.

Metritis-positive *vs.* healthy groups:

Functional category analysis using UP-Keywords incorporated 798 (96.3%) of the genes. Gene ontology (GO) analysis for enrichment of the terms included 701 (84.7%), 561 (67.7%) and 593 (71.5%) genes into cellular component (CC), molecular function (MF) and biological process (BP), respectively. The functional annotation chart for the enriched GO terms for molecular function (MF) with the default options identified 39 different activities (Supplementary Table S9). The molecular function "cytokine activity" (GO: 0005125) included 16 genes (viz. WNT2, growth differentiation factor 15 (GDF15), GDF7, interleukin 17B (IL-17B), IL-2, -22, -36A, -36B, -4, transforming growth factor-beta 2 (TGFB2) etc)

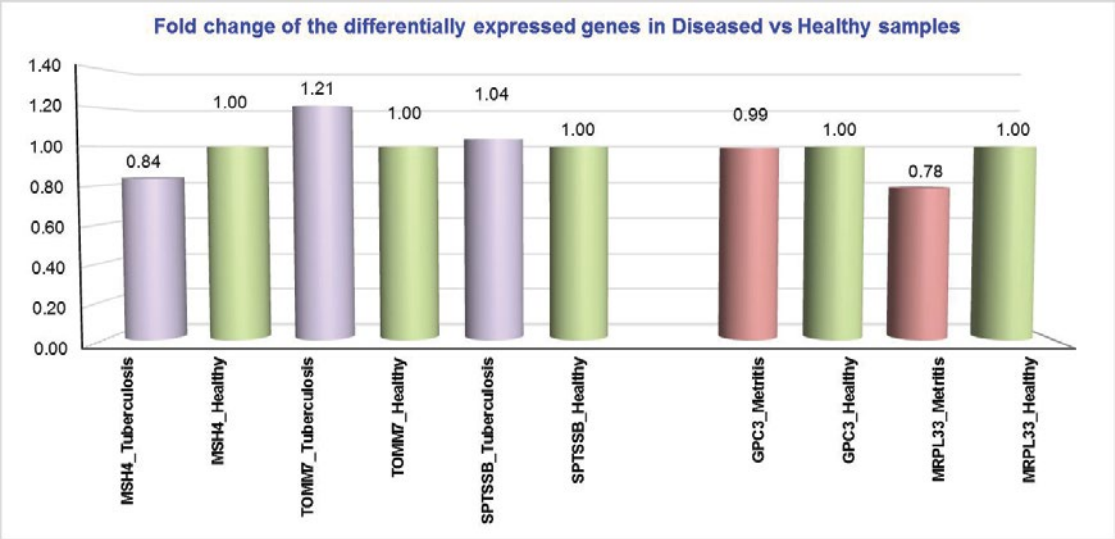

Figure 4. Pie chart of gene ontology of the biological process of the differentially and uniquely expressed protein-coding genes between tuberculosis-positive and healthy groups.

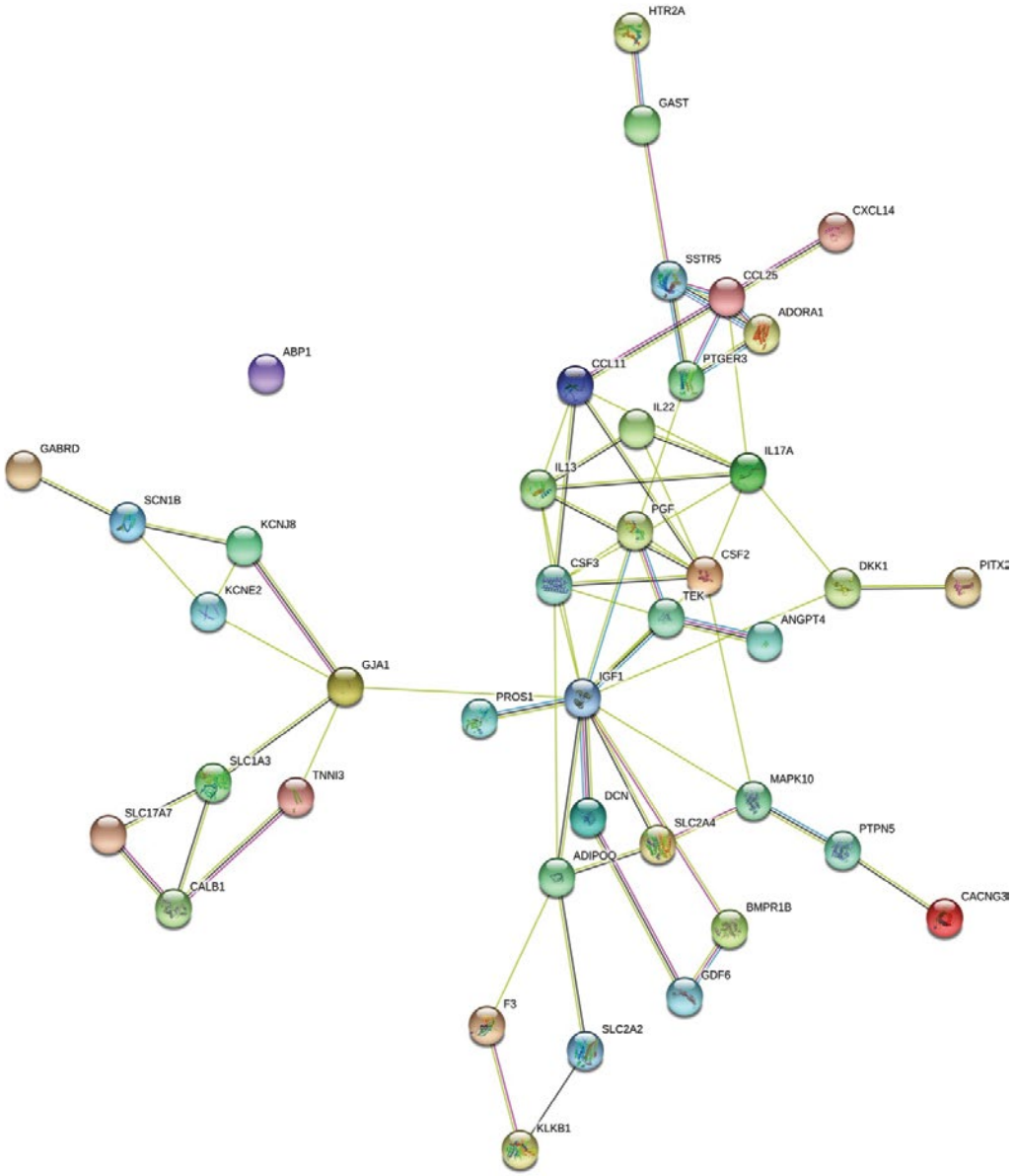

Figure 5. Protein-protein interaction network analyzed by selecting the genes that are intricately involved in the important pathways contributing to resistance or susceptibility to tuberculosis.

(Supplementary Table S10). The molecular functional enrichment analysis identified other activities like transforming growth factor-beta receptor binding (p=2.70, E-04), calcium ion binding (p=4.20, E-04), growth factor activity (p=3.46, E-03), Wnt-protein binding (p=0.005), extracellular matrix binding (p=0.013), hormone activity (p=0.034), and voltage-gated calcium channel activity (0.050).

KEG pathway analysis (279 genes selected by DAVID) with default parameters identified the following important pathways: camp signaling (p=3.47, E-04), Neuroactive ligand-receptor interaction (p=4.51, E-04), Wnt signaling (p=4.92, E-04), TGF-beta signalling (p=8.80, E-04), MAPK signalling (p=0.013), PI3K-Akt signalling (p=0.017), Steroid hormone biosynthesis (p=0.0178), Oxytocin signalling (p=0.0178) pathways (Supplementary Table S11).

Similar types of results have been reported on bovine metritis. The result of a whole-genome association study (GWAS) 671 human subjects in Thailand identified a chromosome 1p13 association (rs1418425, p=2.54, E−8) with TB [50]. This RefSeq variant is located adjacent to the gene encoding the leukocyte surface glycoprotein CD53 which has been correlated with active Tuberculosis [50]. Another GWAS suggested that another important pathway is the innate immune type-I interferon signaling cascade that carries circulating biomarker-signature of pulmonary as well as macrophage response to mycobacterial infection. Upon progression of TB infection, a fine-tune between two pathways, namely, mitogen-activated protein kinase (p38 MAPK) [51] and phosphatidylinositol 3-kinase (PI3-K) signaling controls the neutrophil chemotaxis. The P13-K pathway has immense importance in neutrophilia, occurrence, and progression of TB [47]. The directional migration of 'phosphatidylinositol 4, 5-bisphosphate' and 'phosphatidylinositol-trisphosphate' is modulated by the PI3-Kpathway [52-54]. However, these genes of P13-K pathway (PI3-K δ, AKT, mTORC1, and MNK) are down-regulated during early infection through a set of microRNAs [10,55]. Jurado et al. demonstrated that CD3+PD-1+ lymphocytes are increased in peripheral blood in TB patients [56]. The PD1 plays a central role in host-pathogen interaction by interfering with T cell effector functions against *Mycobacterium tuberculosis*. Another pathway that is controlled by histone deacetylase has been associated with resistance to *M. tuberculosis* infection and this pathway is critical to confer innate immunity against early infection [57]. In bovines, the TNFSF8/TNFRSF8 pathway has been reported to augment the IFγ production upon *M. bovis* BCG stimulation [58].

Functional annotation clustering was performed by DAVID using 829 taurine genes (selected by DAVID) under classification stringency and default threshold parameters. The results showed 61 clusters with the highest Enrichment Score of 4.93 (Supplementary Table S12).

Panther analysis of the DEGs for Metritis-positive and healthy control groups detected 1341 process hits (falling in 13 categories) (Figure 6).

The protein-protein interaction network was constructed using String DB with 29 genes (protein-Ids converted by g:Profiler) selected from the important pathways (PPI enrichment p-value <1.0, e-16; mean local clustering coefficient=0.66) from the analysis discussed above (Figure 7) (Supplementary Table S13). Salilew-Wondim and team investigated the effect of clinical and subclinical endometritis on the endometrial pathways and unveiled the molecular signatures in 42–60 days postpartum Holstein Frisian cows [59]. The endometrial transcriptomic (using GeneChip® Bovine Genome Array) and miRNome (by Exiqon microRNA PCR Human Panel arrays) profiling revealed different expression level of 203 genes in diseased animals. They reported that 92 genes (viz. PTHLH, INHBA, DAPL1, and SERPINA1) and 111 genes (viz. MAOB, CXCR4, HSD11B and, BOLA) were significantly up-regulated down-regulated, respectively, in clinical endometritis group. Gene annotation analysis revealed the most affected pathways and molecular mechanisms were the immune system process, cell adhesion, regulation of apoptotic signaling pathway, Enzyme-linked receptor protein signaling, and G-protein coupled receptor (GPCR) signaling pathways, neurogenesis, and chemotaxis in endometritis animal groups. NF-κB, a key molecule to initiate signaling cascade against inflammatory diseases. Zhang et al. used a mouse model to elucidate the molecular pathways and determine the anti-inflammatory effect of IFN-τ on endometritis caused by Staphylococcus aureus [60]. They reported that IFN-τ inhibited the phosphorylation of IκB, NF-κB p65, and MAPKs (p38, JNK, and ERK). This further prompts the activation of MyD88-dependent signaling pathways that finally induces MAPK activation. The activation of TLR2 due to bacterial infection initiates a cascade of molecular

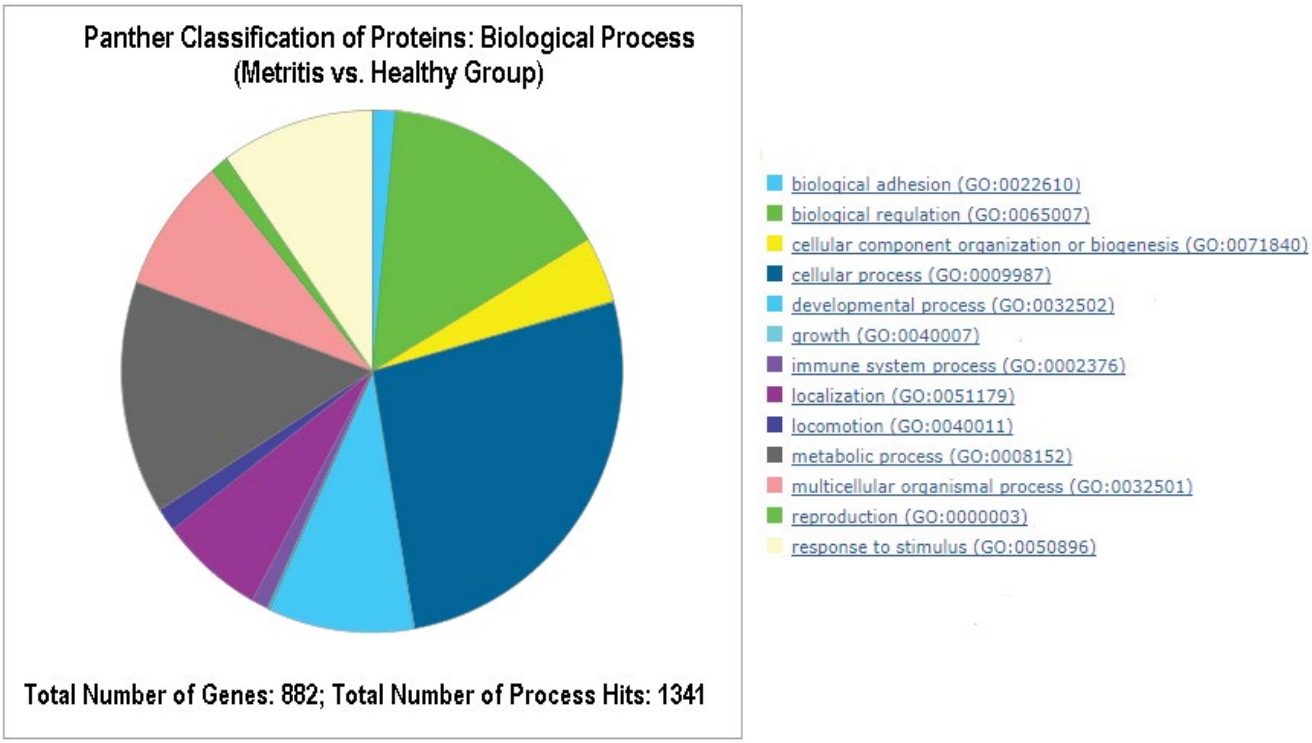

Figure 6. Pie chart of gene ontology of the biological process of the differentially and uniquely expressed protein-coding genes between metritis-positive and healthy groups.

mechanisms, viz. activation of the innate inflammatory response which further induces the NF-κB and MAPK pathways to affect the release of pro-inflammatory cytokines. Besides, TNF-α can contribute to the production of pro-inflammatory cytokines by activating the intracellular IκBα and JNK signaling pathways through TLRs [61-63].

Conclusion

In this study, we have identified the differentially expressed (Log2 FPKM ratio >2 and <2) mRNA genes between diseased (Tuberculosis, Metritis) versus healthy (control) Nili Ravi buffaloes. The gene ontology and pathway analysis associated with the diseased and control group revealed that cytokine activity, transforming growth factor-beta receptor binding, Wnt signaling, Cytokine-cytokine receptor interaction, PI3K-Akt signaling, MAPK signaling and GMP-PKG signaling pathways related genes play important role in TB susceptibility. Similarly, the important pathways associated with metritis-susceptibility are cAMP signaling, Neuroactive ligand-receptor interaction, Wnt signaling, TGF-beta signaling, MAPK signaling, PI3K-Akt signaling. Further studies aimed at targeting these modular genes could be used effectively in the future for marker-assisted selection for better tolerance against these economically devastating diseases in buffaloes.

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