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Integrating Analysis of Publicly Available Microarray Data to Study the Immune Response of Cattle to Infection with *Mycobacterium bovis*

Research Article

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ABSTRACT

Bovine tuberculosis is one of the serious public health challenges which also causes economic damage in the livestock industry. Understanding the interaction mechanism between the host immune system and the causative pathogen of tuberculosis is one of the essential areas of study for success in designing effective drugs to treat tuberculosis. Here, we used four publicly available microarray data to light up the response of the cattle immune systems to *Mycobacterium bovis* at the gene level. Our integrating analysis results on microarray data led to identifying 189 (160 up- and 29 down-regulated) differentially expressed genes for infected samples with *Mycobacterium bovis* against uninfected samples. Gene ontology and pathway analysis indicated that most of the differentially expressed genes are related to the immune system's (especially innate immune system) response to the pathogen. Finally, 122 proteins (108 up-regulated and 14 down-regulated) were included in the constructed protein-protein interaction network among the proteins from differentially expressed genes. We identified 11 genes as hub genes based on three methods using the cyto Hubba plug-in in the Cytoscape. Based on our analysis, most differentially expressed genes are related to the innate immune system. However, considering the impact of time on the microarray data analysis indicated that associated gene expression with the adaptive immune system increased by time.

KEY WORDS cattle, cytokine, immune system, macrophage, microarray, *Mycobacterium tuberculosis*.

INTRODUCTION

Bovine tuberculosis is a zoonotic disease, caused by *Mycobacterium bovis*, is one of the most critical diseases in terms of economic losses and threats to public health (Ejeh *et al.* 2014). Annually, more than 50 million cattle become infected with *Mycobacterium bovis* globally, and the economic cost of this disease is about $3 billion (Waters and Palmer, 2015). Although cattle are the primary hosts of *Mycobacterium bovis*, however, this bacterium is still responsible for about 5% of tuberculosis in humans (Michel *et al.* 2010). Tuberculosis is a deadly disease that killed about 1.7 million people in 2016 (Alam *et al.* 2019).

Macrophages are essential components of the innate immune system against tuberculosis-causing agents such as *Mycobacterium bovis*. These intracellular pathogens have acquired some abilities to overcome their host immune systems during evolution. The success of these pathogens depends on factors such as their ability to survive and proliferate inside infected macrophages (Philips and Ernst, 2012). The most important strategy of these bacilli to survive inside macrophages is to manipulate the phagocytosis process and prevent the normal maturation of phagosome into the acidic and hydrolytic components. Through this, pathogens are protected from the direct access of the immune system (Russell, 2001).

One of the most effective ways to help researchers design effective drugs to treat tuberculosis is to study gene interaction between pathogens and the host immune system. Several studies (Killick *et al*. 2011; Magee *et al*. 2012; Caimi *et al*. 2013; Killick *et al*. 2014) have investigated gene expression differences in the macrophages and other immune cells infected with *Mycobacterium bovis* in cattle. In this study, four publicly available data of the gene expression omnibus (GEO) database have been combined to recognize differentially expressed genes (DEGs) of cattle immune systems in response to *Mycobacterium bovis*. Also, pathway enrichment analysis, gene ontology (GO), protein-protein interaction (PPI), and detection of hub genes construction were carried out for detected DEGs.

MATERIALS AND METHODS

Obtaining and pre-processing microarray data

To identify studies profiling the response of cattle immune system to *Mycobacterium bovis* infection, we searched the National Center for Biotechnology Information (NCBI) GEO database (http://www.ncbi.nlm.nih.gov/geo/).

Mycobacterium bovis was used as a search keyword, and filtration was done based on the organism (*Bos taurus*), study type (expression profiling by array), entry type (Dataset/Series), and platform (Affymetrix Bovine Genome Array, GPL2112). Based on the mentioned criteria, 4 microarray data sets (GSE33309, GSE33359, GSE39819, GSE59774) were selected, and their CEL files were downloaded for downstream analysis (Table 1).

After downloading the mentioned datasets, we found that 21 samples are common between GSE33309 and GSE59774 datasets. A series of duplicate data were removed to avoid bias in the analysis. Data reading and normalization of each experiment were done using a robust multi-array average (RMA) algorithm in the R affy package (Gautier *et al*. 2004). It should be noted that due to the corruption in 4 files (GSM979839, GSM979840, GSM979841, GSM979842) related to the GSE39819 dataset, we used the deposited gene expression matrix of the GSE39819 dataset (which contained all 10-samples) instead CEL files. In the next step, the quality test was performed using the array quality metrics package (Kauffmann *et al*. 2009) for all samples based on normalized datasets. Finally, 95 samples were used in the final data set. Affymetrix bovine annotation data (Bovine.db) Bioconductor package was used to annotate (adding official gene symbol and Entrez ID) each normalized dataset.

Identification of differentially expressed genes

After data integration of 4 sets, batch effect removal was done using ComBat in sva R package.

The batch effect represents the non-biological and systematic technical variations that arise during sample processing and measurement at different times, places and, conditions (Lazar *et al*. 2013).

Principal component analysis (PCA) was performed before and after batch effect removal to visualize clustering patterns of studied sets and samples (Figure 1). LIMMA package (Phipson *et al*. 2016) was performed to identify differentially expressed genes. Cut-off criteria for final selection of DEGs defined as false discovery rate (FDR) adjusted p-value < 0.05 and $|\log2 \text{ Fold change}| \geq 0.58$ (Fold change$\geq$1.5) (Figure 2). Also, based on PCA results, we divided samples into three groups, including G1 (healthy samples), G2 (infected samples 2-6 hours after the infection, as well as infected samples which the time after infection was not specified for them), and G3 (infected samples 24 hours after infection). We compared G2 (43 samples) *vs*. G1 (38 samples) and G3 (14 samples) *vs*. G1 to identify DEGs. GO analysis was performed on DEGs to understand the time effect on gene expression profile for the classified samples.

Functional analysis

We subjected the list of down- and up-regulated genes for the KEGG pathway and GO analyses using DAVID (Sherman and Lempicki, 2009). The results of GO analysis were categorized into three classes, including Biological Process, Cellular Component, and Molecular Function. Benjamini-Hochberg correction was used for calculated p-values of the multiple testing, and p-adjusted < 0.05 was considered a significant level.

Construction PPI network and identification of hub genes

The STRING app from Cytoscape (Shannon *et al*. 2003) software was used for PPI network construction of DEGs. In the constructed network, nodes edges represent the proteins and the interaction between them, respectively, and the labels of nodes indicate the official symbols of the identified DEGs.

PPI networks facilitates the understanding and interpreting of the relationship between proteins in a complex biological process. cytoHubba (Chin *et al*. 2014) plug-in in the cytoscape was used to identify hub genes, commonly identified genes using maximal Clique Centrality (MCC), maximum neighborhood component (MNC), and degree (based on the number of interactions per gene) methods, were considered as hub genes in this study. Hub genes are defined as genes with a highly connected to other genes in a PPI network. Since each extensive network may have contained several sub-networks, we used the MCODE plug-in in the Cytoscape to identify denser sub-network.

Table 1 Summary of datasets presented in this study

Experiment	Arrays	Infected samples	Control samples	Reference
GSE33309	49	21	28	Dave *et al.* (2002)
GSE33359	16	8	8	Caimi *et al.* (2013)
GSE39819	10	8	2	DesJardin *et al.* (2002)
GSE59774	20	20	-	Ejeh *et al.* (2014)

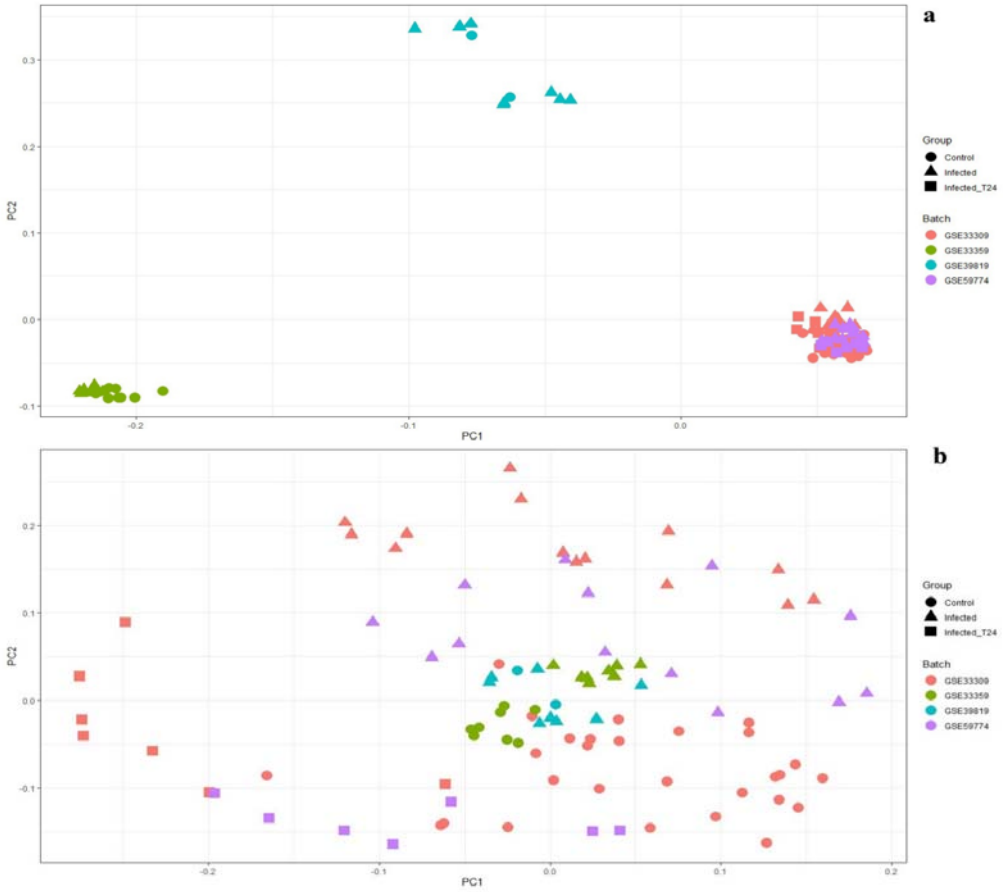

Figure 1 PCA results for studied samples before (a) and after (b) batch effect removal. Control, infected and infected_T24 are classified as G1, G2, and G3, respectively

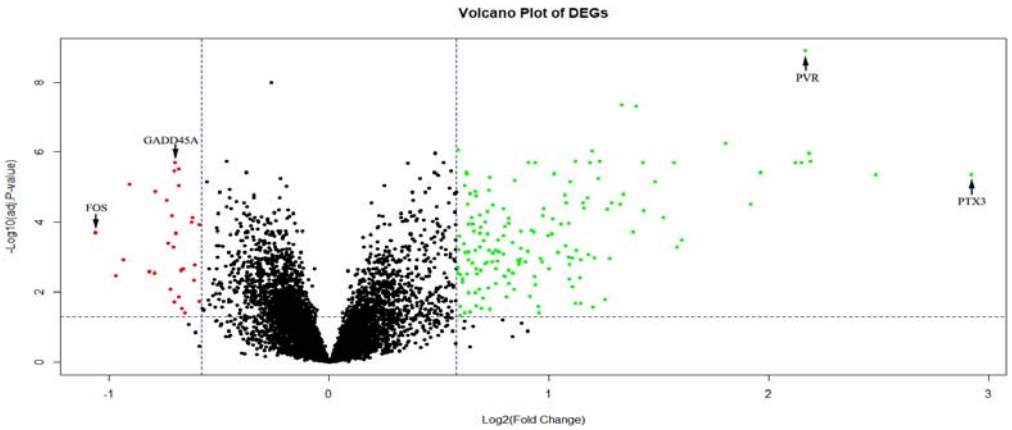

Figure 2 Volcano plot showing the DEGs between infected and healthy samples. Green (up-regulated) and red (down-regulated) dots indicate DEGs with adjusted P-value < 0.05 and |log2 Fold change| ≥ 0.58 (Fold change≥1.5). The black dots fail to meet our criteria. Genes with the highest fold change and lowest adjusted p-value were marked for up- and down-regulated genes

Network clustering was performed based on topology to find densely connected regions. Then, a Cytoscape plug-in named CluGO (Bindea *et al*. 2009) was carried out for GO analysis and to visualize functionally grouped terms of the obtained sub-network. GO analysis allows us to understand better and express the biological function of genes and it classifies them into three categories, including biological process (BP), molecular function (MF), and cellular component (CC) (Khalkhali-Evrigh *et al*. 2022).

RESULTS AND DISCUSSION

After data collection and running the pre-processing on them, finally, we reached 95 samples which comprised 57 infected samples and 38 healthy individuals as control samples. A total of 189 DEGs were identified after analyzing all mentioned samples (infected *vs*. control), of which 160 were up- and 29 were down-regulated genes. The fold change of up-regulated genes ranged from 1.50 for the SPX gene to 7.57 for the PTX3 gene, while this range for down-regulated genes was from 1.50 for the CD101 gene to 2.09 for the FOS gene. Also, based on the statistic parameter (adjusted P-value), the most significant up- and down-regulated genes were the PVR gene (adjusted P-value equal to 1.27E-09) and the GADD45A gene (adjusted P-value equal to 2E-06), respectively.

Significantly enriched terms from GO analysis on up-regulated genes in BP, CC, and MF categories were 19, 2, and 5 terms, respectively (Table 2). With the mentioned criteria in the material and methods section, we found no significantly enriched terms for down-regulated genes.

The number of identified DEGs for G2 *vs*. G1 and G3 *vs*. G1 that passed our criteria were 262 (213 up- and 49 down-regulated genes) and 627 (394 up- and 233 down-regulated genes), respectively. Also, the number of shared DEGs between the G2 *vs*. G1 and G3 *vs*. G1 was 42 (36 up- and 6 down-regulated genes).

Pathway analysis

Obtained results from kyoto encyclopedia of genes and genomes (KEGG) analysis revealed that 34 KEGG pathways significantly enriched for up-regulated genes list (Top ten pathways in Table 3. The KEGG is a collection of manual pathway maps demonstrating our knowledge of the molecular interaction, reaction, and communication networks in different areas of biological science like metabolism, genetic information processing, cellular processes, and organism systems.

In the present study, the most enriched pathways for mentioned genes were associated with the immune system (such as Cytokine-cytokine receptor interaction, TNF signaling pathway, Toll-like receptor signaling pathway, etc.) and disease (such as Rheumatoid arthritis, Salmonella infection, Tuberculosis, etc.).

For enriched pathways, the p-value adjusted based on Benjamin-Hochberg less than 0.05 was considered the significant cut-off value. No significantly enriched pathway was found for down-regulated genes.

PPI network

We used all DEGs (189) for PPI network construction, and 184 genes from this list were included in the network. After removing singleton nodes (nodes without edges) from the constructed network, finally, 122 proteins (belonging to the 108 up- and 14 down-regulated genes) with 804 edges (interaction) were included in the network (Figure 3a). The number of interactions for ten genes with the most interaction is represented in Table 4.

Extracted sub-network from the leading network that was the densest subset consisting of 21 proteins and 204 edges (Figure 3b). The GO analysis of 21 genes present in the sub-networks showed that more than77 percent of enriched terms were classified in four gropes including “cellular response to lipopolysaccharide” (40.78%), “cytokine receptor binding” (20.39%), “granulocyte chemotaxis” (8.74%) and “positive regulation of response to external stimulus” (7.38%; Figure 3c).

The defense response of the immune system against different pathogens like bacteria and viruses is a routine process in organisms. However, studying how the immune system responds at the level of genes and proteins can help us to control diseases by developing effective diagnostic and treatment methods. Inflammation can be defined as a multi-component response of the immune system to different external threats like pathogens (Medzhitov and Horng, 2009).

Macrophages, a prominent part of the innate immune system, are among the first defensive lines against tuberculosis (Queval *et al*. 2017).

Along with the phagocytic activity of macrophages, secretion of chemotactic and inflammatory cytokines, and the beginning of a process called chemotaxis bring together the components of the innate immune system against pathogens (Sokol and Luster, 2015).

Here, the results of GO analysis for DEGs were consistent with the behavior of the immune system against pathogens, so most up-regulated DEGs are enriched in “inflammatory response”, “immune response”, “cellular response to interferon-gamma”, “monocytes chemotaxis” and “defense response to bacterium” in BP category; also “chemokine activity” and “cytokine activity” in MF category. Cellular component analysis revealed that the most productive up-regulated DEGs were localized in the extracellular space and the external side of the plasma membrane of cells.

Table 2 Significantly gene ontology (GO) terms for up-regulated genes

Class	GO term	Description	Genes	P-value
BP (top 15)	GO:0006954	Inflammatory response	22	1.67E-12
	GO:0006955	Immune response	20	1.28E-10
	GO:0070098	Chemokine-mediated signaling pathway	11	3.39E-09
	GO:0071346	Cellular response to interferon-gamma	10	1.12E-08
	GO:0071347	Cellular response to interleukin-1	10	1.19E-07
	GO:0071222	Cellular response to lipopolysaccharide	11	2.19E-07
	GO:0030593	Neutrophil chemotaxis	9	2.11E-06
	GO:0060326	Cell chemotaxis	8	3.97E-05
	GO:0042832	Defense response to protozoan	6	4.98E-05
	GO:0042742	Defense response to bacterium	9	1.15E-04
	GO:0071356	Cellular response to tumor necrosis factor	8	1.26E-04
	GO:0048247	Lymphocyte chemotaxis	6	1.53E-04
	GO:0050729	Positive regulation of inflammatory response	7	2.26E-04
	GO:0070374	Positive regulation of ERK1 and ERK2 cascade	10	3.46E-04
	GO:0044130	Negative regulation of the growth of symbionts in the host	5	3.74E-04
CC	GO:0005615	Extracellular space	39	3.15E-13
	GO:0009897	External side of plasma membrane	12	1.24E-05
	GO:0033256	I-kappaB/NF-kappaB complex	3	0.0381
MF	GO:0008009	Chemokine activity	12	7.67E-12
	GO:0005125	Cytokine activity	13	6.45E-07
	GO:0048020	CCR chemokine receptor binding	6	1.63E-04
	GO:0045236	CXCR chemokine receptor binding	5	1.70E-04
	GO:0008083	Growth factor activity	7	0.0037

BP: biological process; MF: molecular function and CC: cellular component.

Table 3 Top 10 enriched KEGG pathways for up-regulated genes

Term	Genes	P-value
TNF signaling pathway	EDN1, CSF2, IL15, CCL20, CFLAR, TRAF1, PTGS2, CXCL3, GRO1, CXCL2, TNF, NFKB1, ICAM1, PIK3R5, SOCS3, IL6, CCL5, IL1B, LTA, CCL2, BIRC3	2.83E-18
Cytokine-cytokine receptor interaction	IL10, CD40, CSF3, CSF2, IL15, CCL20, CXCL13, TNF, TNFSF13B, IL1A, IL6, LOC510185, IFNG, CCL5, IL1B, CCL4, LTA, CCL3, IL12B, ACKR3, CCL2	4.16E-12
Rheumatoid arthritis	CSF2, IL15, CCL20, MMP1, CD80, TNF, CXCL5, ICAM1, TNFSF13B, IL1A, IL6, IFNG, CCL5, IL1B, CCL3, CCL2	1.13E-11
NF-kappa B signalling pathway	CD40, CFLAR, TRAF1, PTGS2, TNF, NFKB1, RELB, ICAM1, NFKB2, TNFSF13B, IL1B, CCL4, LTA, LAT, BIRC3	8.29E-11
Salmonella infection	IL1A, IL6, CSF2, IFNG, NOS2, IL1B, CCL4, CCL3, CXCL3, GRO1, CXCL2, NFKB1	5.96E-09
Malaria	SELP, IL10, CD40, CSF3, IL6, IFNG, GYPC, IL1B, CCL2, TNF, ICAM1	1.17E-08
Chagas disease	IL10, NOS2, CFLAR, TNF, NFKB1, PIK3R5, IL6, IFNG, CCL5, IL1B, CCL3, IL12B, CCL2	1.49E-08
Legionellosis	IL6, IL1B, IL12B, GRO1, CXCL3, CXCL2, TNF, NFKB1, NAIP, NFKB2	1.88E-08
Toll-like receptor signalling pathway	CD40, IL6, CCL5, IL1B, CD80, CCL4, CCL3, IL12B, TNF, NFKB1, PIK3R5	6.49E-07
Amoebiasis	IL10, ARG2, IL6, CSF2, IFNG, NOS2, IL1B, ACTN1, IL12B, TNF, NFKB1, PIK3R5	1.15E-06

In the present study, of the 10 most up-regulated genes, the PTX3 gene was in the first rank (Fold change equal to 7.56). Based on an RNA-Seq study on humans, this gene is mainly expressed in the bone marrow tissue (Fagerberg *et al*. 2014).

The product of this gene increases in response to inflammation and stress, and interacting with pathogens and damaged host cells help the innate immune system remove them (Balhara *et al*. 2013).

Two (CCL20 and CXCL2) and four genes (IL1-a, IL1-b, IL6, and TNF) from the mentioned list belonged to chemokines and cytokines, respectively. CCL20, and CXCL2 were produced by macrophage and mast cells and induced the recruitment and migration of innate immune cells to infected tissues (Sokol and Luster, 2015). Production of IL1-b by macrophages due to its antimicrobial properties is considered an effective strategy to prevent disease development (Lee *et al*. 2019).

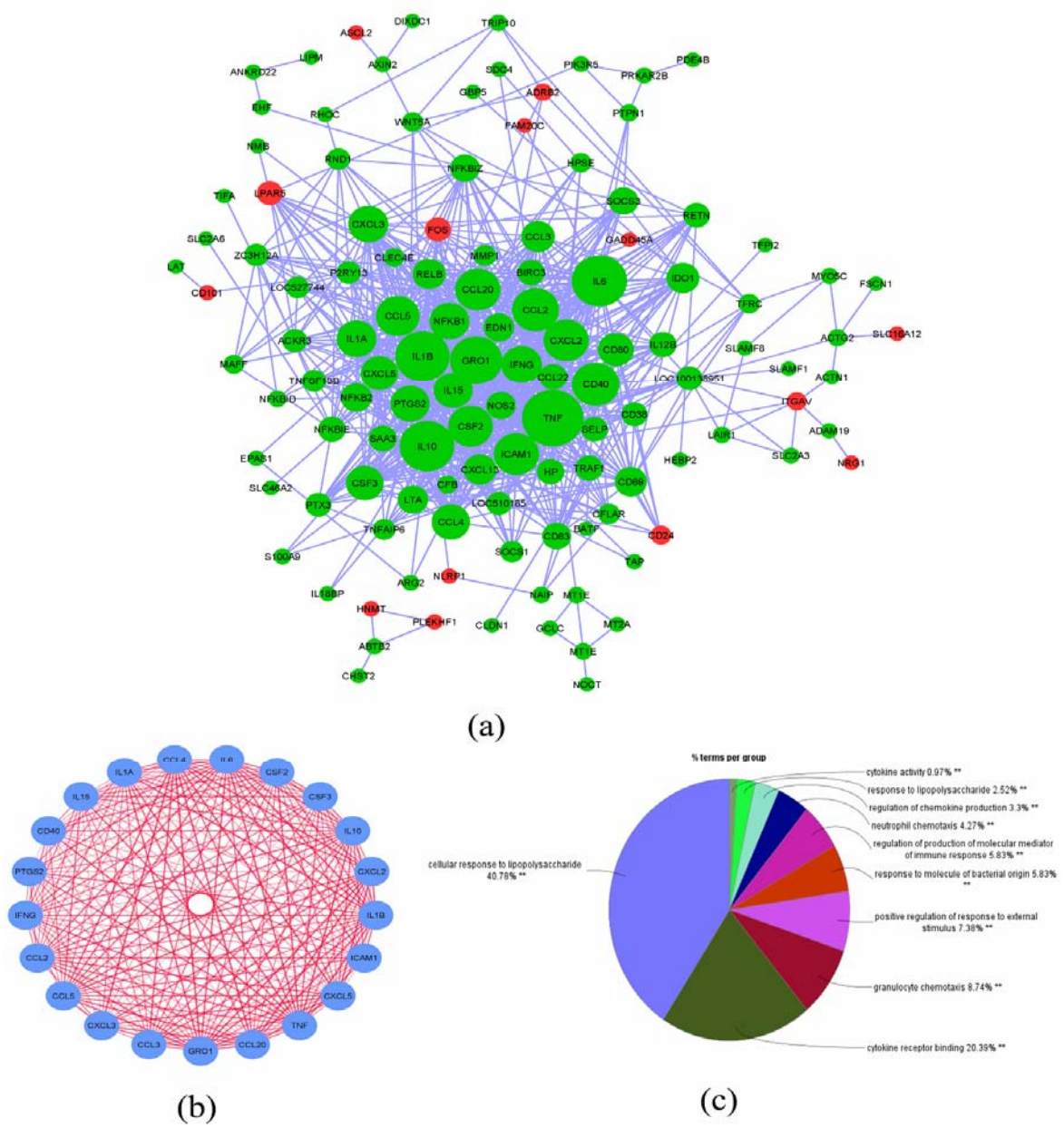

Figure 3 DEGs PPI network construction. (a) Constructed PPI network for DEGs including 108 up- (green) and 14 down-regulated (red) genes; the size of nodes is based on the number of connections; larger nodes have more edges. (b) Extracted sub-network from the main PPI network including 21 nodes. (c) Grouped results of GO analysis for 21 genes included in the sub-network

Table 4 Ten top genes with the most interaction in the constructed protein-protein interaction (PPI) network

Gene	Description	Position	Number of edges
TNF	Tumor necrosis factor	Chromosome 23	60
IL6	Interleukin 6	Chromosome 4	52
IL1B	Interleukin 1 beta	Chromosome 11	51
IL10	Interleukin 10	Chromosome 16	51
GRO1	Chemokine (C-X-C motif) ligand 1	Chromosome 6	48
CD40	CD40 molecule	Chromosome 13	42
CCL2	chemokine (C-C motif) ligand 2	Chromosome 19	41
ICAM1	Intercellular adhesion molecule 1	Chromosome 7	39
CXCL2	chemokine (C-X-C motif) ligand 2	Chromosome 6	39
CCL20	C-C motif chemokine ligand 20	Chromosome 2	39

However, there is evidence to show that its long-term production can lead to host tissue damage (Zhang *et al*. 2014).

The PVR gene, along with interaction with other genes like DNAM-1 promotes monocyte transendothelial migration (Reymond *et al*. 2004; Gerhardt and Ley, 2015), as a prerequisite for innate immune system response to pathogens. The encoded protein by the *TFPI2* gene can play an essential role in inhibiting serine proteases. Studies on *Mycobacterium tuberculosis* have shown that this pathogen employs serine protease as a virulence factor to damage and disable the host immune system (Dave *et al*. 2002; Naffin-Olivos *et al*. 2017). Therefore, it is possible that increasing the expression of the *TFPI2* gene suppresses this ability in the bacteria.

Pathway analysis for up-regulated genes revealed mostly enriched terms related to disease (like Tuberculosis) and immune system response. In the present study, some crucial enriched pathway terms were "TNF signaling pathway", "Cytokine-Cytokine receptor interaction", "NF-kappa B signaling pathway" and "Toll-like receptor signaling pathway". In response to mycobacterial pathogens, macrophages produce toll-like receptors (TLRs) to interact with pathogen-associated molecular patterns (PAMPs) and induce TNF secretion (Killick *et al*. 2013).

One of the essential roles of TNF as a pro-inflammatory cytokine is inducing apoptosis and necroptosis in infected macrophages as a preventive strategy against the development of infection (Xu *et al*. 2014). The NF-kappa B signaling pathway is considered a key and central to the immune system response to many pathogens like mycobacteria. This pathway is activated through pattern-recognition receptors (PRRs) expressed in response to PAMPs and responsible for stimulating the production of cytokines, chemokines, adhesion molecules, and many other molecules required by the innate and adaptive immune system (Liu *et al*. 2017).

We observed that the number of immune-related genes with a significantly different expression between infected and control samples increased over time so that DEGs in G2 *vs*. G1 and G3 *vs*. G1 were 262 and 627, respectively. Comparison of GO and pathway analysis results showed that one of the exciting differences between the two groups was the enriched term "antigen processing and presentation of endogenous peptide antigen via MHC class I" in the biological process category and "Antigen processing and presentation" in the KEGG pathway for G3 *vs*. G1.

During antigen processing and presentation as an immunological process, foreign proteins are digested into peptide fragments and transported to the surface of antigen-presenting cells (APCs) for recognition by T cells. Major histocompatibility complex (MHC) class I molecules form a complex with antigen peptides in the endoplasmic reticulum to export to the plasma membrane (Abele and Tampe, 2009). It seems MHC class I is a critical factor against tuberculosis, so disruption of MHC class I antigen processing made the studied mice more susceptible to *Mycobacterium tuberculosis* infection (Urdahl *et al*. 2003). Also, evidence suggests that MHC class I serve as a target for suppression by *Mycobacterium tuberculosis* (Meng *et al*. 2017). Since antigen presentation by MHC proteins plays a vital role in adaptive immunity (Wieczorek *et al*. 2017), it seems, over time, genes associated with the innate immune system are replaced by genes that light up the adaptive immune system for a more efficient defeat of *Mycobacterium bovis*. While, results of GO analysis revealed that in the early hours of infection (G2), genes associated with GO terms such as "cell chemotaxis", "cytokine activity", "chemokine activity" and "CXCR chemokine receptor binding" have significantly changed in expression level. Chemokines are chemotactic cytokines that play various vital roles in immune system function (Griffith *et al*. 2014), especially in the first hours of infection.

All 11 identified hub genes (CCL20, CD40, CSF2, CXCL2, GRO1, ICAM1, IFNG, IL1b, IL6, IL10, and TNF) belonged to up-regulated genes. The product of the CD40 gene is a transmembrane protein that acts as a receptor on APCs. Its interaction with CD40L (ligand on T cells) activates macrophage and other APCs antimicrobial properties (Méndez-Samperio *et al*. 2003) it has a vital role in immunity against intracellular pathogens (Gurunathan *et al*. 1998) like *Mycobacterium bovis*. CSF2, also known as a granulocyte-macrophage colony-stimulating factor (GM-CSF) is expressed by nonhematopoietic and hematopoietic cells and restricts the development of tuberculosis (Robinson, 2017). Following our study, several studies have shown that ICAM1 expression increases after infection with *Mycobacterium bovis* and tuberculosis (DesJardin *et al*. 2002; Li *et al*. 2017).

One of the crucial roles reported for ICAM1 expressed on APCs is facilitating leukocyte transmigration across the endothelium (Müller, 2019) as an essential part of the immune system's response to pathogens. IL6 which is produced by T cells and macrophages is probably involved in the secretion of IFNG (Goovaerts *et al*. 2013). IFNG is a cytokine that mediates the activation of macrophages to kill intracellular pathogens and the production of other cytokines (Flynn *et al*. 1993).

CONCLUSION

In the present study, we combined four publicly available microarray data to integrate separately conducted studies. Analysis of the mentioned data led to the detection of 160 up- and 29 down-regulated genes. These genes were sig-

nificantly enriched in immune-related GO terms and pathways. We highlighted 11 genes defined as hub genes based on three methods, including MCC, MNC, and the number of interactions (degree). Most identified hub genes as cytokines and chemokines play a role in innate immune system response to pathogens. Also, the investigation of the effect of time on the pattern of gene expression in infected samples showed that associated gene expression with the adaptive immune system increase over time. Some of the obtained results in this study can be the basis for designing experiments to understand better the host (cattle) immune response to *Mycobacterium bovis*. The genes identified in this study can be used as genetic markers to identify the mentioned disease and design effective drugs. However, more studies are needed to confirm these results.

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