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Uncovering the molecular mechanisms of bovine tuberculosis through meta-analysis of differentially expressed genes

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ABSTRACT

Bovine tuberculosis (BTB) is a chronic bacterial disease, caused by *Mycobacterium bovis.* It is a globally significant disease affecting cattle production and health. In the present meta-analysis study, alveolar macrophage tissue from three independent studies was analyzed, comprising 21 biological replicates (100 RNA-Seq data) from BTB infected and non-infected cattle. RNA-Seq analysis was performed on individual studies to obtain count matrices, followed by meta-analysis using one-sided NOISeq method. Fisher's and Stouffer's methods yielded 5904 and 2631 DEGs respectively. Functional annotation and pathway enrichment analysis revealed key biological, molecular, and cellular functions involved in pathogenesis. A protein interaction network identified ten hub genes that were mainly associated with the proteasome complex playing role in host immune response. JAK/STAT signaling pathway, Notable pathways included Pentose phosphate pathway, Ubiquitin proteasome pathway, Toll receptor signaling pathway, Interleukin signaling pathway among the 36 enriched pathways. The findings highlighted the role of immunological pathways and genes upregulated during BTB in cattle. Meta-analysis improved statistical power and robustness compared to individual studies, offering insights into potential biomarkers compared to individual studies. The findings enhance the understanding of BTB pathogenesis and offer direction to bovine breeders in endeavour of improved production and management.

Keywords: Bovine tuberculosis, Cattle, Hub genes, Meta-analysis, Transcriptome

INTRODUCTION

Bovine tuberculosis (BTB) is caused by *Mycobacterium bovis*, belonging to the *Mycobacterium tuberculosis* complex and affects various mammals, especially cattle. It is contagious and spreads through infected animals, inhaling infectious droplets, or by drinking unpasteurized milk from sick calves. It contributes to about 10% of human tuberculosis cases in some regions (World Organisation for Animal Health) and remains a serious issue in tropical and sub-tropical countries (Borham *et al.* 2022). Globally, it is the fourth most significant cattle disease with about 50 million infected cattle causing annual losses of $3 billion (Abdelaal *et al.* 2022). In India, BTB is endemic affecting an estimated 7.3% cattle with increasing numbers due to intensified dairy farming practices (Ramanujam and Palaniyandi 2023). BTB causes reduction in milk (10–12%) and meat production (6–12%), higher death rates, and lower fertility rates causing substantial economic losses (Tschopp *et al.* 2021).

Advances in transcriptomics technology (RNA-Seq) facilitated understanding of the host response mechanisms to *M. bovis* infection by revealing genes responsible for immune responses (Garg *et al.* 2024). For instance, transcriptome studies involving peripheral blood and macrophage tissues in BTB infected and healthy cattle and across different time points of infection have been performed to identify biomarkers associated with *M. bovis* infection in cattle (Blanco *et al.* 2023). Since the mycobacterium invades the host alveolar macrophage and evades the host immune system, understanding the host-pathogen interactions during infection is important to uncovering the disease mechanism. Molecular host-pathogen interaction studies generated vast data, but variability in sample size, design, and biological variances complicates identifying common gene expression patterns. Meta-analysis overcomes this by integrating data from multiple studies, enhancing sample size and statistical power to reveal BTB-related gene expressions (Vijayakumar *et al.* 2019). This study performs a meta-analysis on the transcriptome of 100 RNA-seq data across three studies involving macrophage tissue from infected and healthy cattle across diverse ages and sexes. By identifying conserved gene expression patterns and molecular pathways across diverse populations, this research aims to provide a comprehensive understanding of BTB pathophysiology and host immune responses.

MATERIAL AND METHODS

Data selection and retrieval: A comprehensive search

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was performed to find RNA-seq studies profiling gene expression to understand molecular mechanism underlying bovine tuberculosis in cattle. Three independent RNA-Seq studies involving BTB infected and healthy macrophage tissue from *Bos taurus* were obtained from NCBI, providing a total of 21 biological replicates for meta-analysis. The study details are outlined in Table 1.

RNA-Seq analysis: For maintaining the coherence and reliability of data, three RNA-Seq studies were analyzed by a uniform, standardized bioinformatics pipeline. Raw reads were assessed with *FASTQC* (Andrews 2016) followed by trimming of adapters, removal of low-quality bases and short reads using the *Trimmomatic* (v0.39) (http://www.usadellab.org/cms/?page=trimmomatic) tool. The cleaned reads were aligned to the ARS-UCD1.3 (GCF_002263795.2) cattle reference genome using *HISAT2* (Kim *et al.* 2019) retaining samples with unique mapping rates > 90%. The reads were assembled using *StringTie v2.1.4* (Pertea *et al.* 2016), incorporating the reference genome for accurate gene annotation. The merge-stringtie mode created a unified, non-redundant set of transcripts across all samples. Transcript abundance was estimated with *RSEM* (v1.3.1) (Li and Dewey 2011), generating count matrices and normalized read counts in Transcripts Per Million (TPM) accounting for sequencing depth and gene length variation that allows accurate comparisons of expression levels between samples. Annotation of transcript ids from *StringTie* was performed using the NCBI's *Batch Entrez* tool.

Meta-analysis for identification of differentially expressed genes: The count matrices from each study were utilized for transcriptome meta-analysis. Transcripts with zero counts across all sample were filtered, and the remaining transcripts were analysed using the one-sided NOISeq method in the *MetaSeq* R package (Tsuyuzaki and Nikaido 2021). The transcript abundance was normalized across studies using TMM (Trimmed mean of M-values) method. The parameters used for meta-analysis include k = 0.5 (number used to replace zero counts), nss = 5 (numbers of samples required to simulate for each condition), v = 0.02 (variability in each simulated sample) and lc = 1 (length correction). The differentially expressed genes (DEG) were identified using Fisher's and Stouffer's method with p-value <0.05. Fig.1 shows the overall methodology used in the study.

Functional annotation, pathway analysis and PPI network construction: Functional annotation and pathway analysis were performed using the *Panther Classification System* (https://www.pantherdb.org/). Fisher's exact test with FDR correction ($p < 0.05$) identified significantly enriched GO terms (biological processes, molecular functions, and cellular components) and pathways.

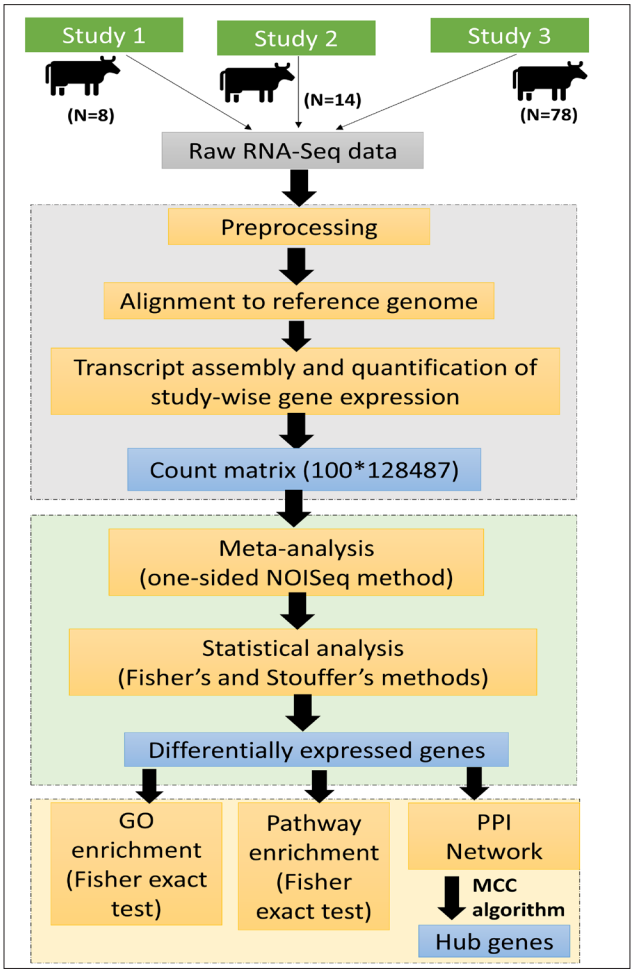

Fig.1. Over-all methodology used in the meta-analysis study

The Protein-Protein Interaction (PPI) network was built using the *STRINGdb* (v12.0) R package, mapping DEGs to the *Bos taurus* reference genes. Using this package, we identified and mapped potential interactions between the proteins encoded by the DEGs. *Cytoscape* (https://cytoscape.org/) was used to visualize the PPI network and *CytoHubba* (https://apps.cytoscape.org/apps/cytohubba) plugin was employed to identify most significant hub genes using the Maximal Clique Centrality (MCC) algorithm. "STRING enrichment" module of *Cytoscape* further

Table 1. Description of the selected transcriptome studies

BioProject ID	No. of RNA-Seq data	Number of biological replicates	gender	breed	Developmental stage	Sequencing Instrument	Library layout	Reference
PRJNA480009	8	4	F	Holstein–Friesian	4 months	Illumina HiSeq 4000	paired	(Hall *et al.* 2020)
PRJNA264301	78	10	M	Holstein–Friesian	7-12 weeks	Illumina HiSeq 2000	paired	(Nalpas *et al.* 2015)
PRJNA194043	14	7	F	Holstein–Friesian	4 Years	Illumina Genome Analyzer IIx	single	(Nalpas *et al.* 2013)

Table 2. Information on RNA-seq data from three BTB studies

BioProject ID	Average number of reads (million)	Average mapping rate (%)	Number of transcripts	Num of DEGs in reference study	p-value used in reference study
PRJNA480009	39.2	96.93	95381	7757	0.1
PRJNA264301	19.5	98.17	132259	95 (2h), 1290 (6h), 5515 (24h), 7321 (48 h)	0.05
PRJNA194043	9.95	93.57	95335	2,584	0.05

analyzed functional associations.

RESULTS AND DISCUSSION

The RNA-Seq analysis produced an average 29.35 million paired-end and 9.9 million single-end reads with an average mapping score of 96%. The combined count matrix consisted of 148383 transcripts in total which after filtering for lowly expressed genes resulted in 128487 transcripts that showed expression in at least one study. Table 2 summarizes the RNA-Seq analysis results and number of DEGs in the reference studies. PCA analysis of the normalized gene expression values explained 80% of the variation with study differences being the primary source (Supplementary Fig.1) along with clear separation between BTB-infected and non-infected cattle across all three studies.

In order to increase statistical power and identify true DEGs, meta-analysis of the three studies was performed using One-sided NOISeq method specifically designed for meta-analysis of RNA-seq data, as it handles read size effect; a bias that can occur in meta-analysis due to differences in sequencing depths across studies. It does not assume a specific data distribution thus allowing for flexibility in capturing complex data distributions from multiple studies. Differential expression analysis between BTB infected and non-infected cattle using Fisher's and Stouffer test identified upregulated and downregulated genes with a probability ≤ 0.05. Stouffer's test combines z-scores obtained from p-values rather than directly combining p-values, whereas Fisher's approach combines p-values from separate tests into a single test statistic (Alves and Yu 2014). A total of 5904 genes were identified as DEGs by Fisher's test of which 5868 genes were upregulated in the infected cattle while 36 were downregulated. Stouffer's test identified 2631 genes as upregulated while no genes were identified as downregulated. A total of 2520 genes were commonly upregulated by both methods including 8 unannotated or novel transcripts. Notable genes such as *IL1B*, *FTH1*, *COX1*, *CXCL5, CXCL8* and *MMP12* showed high expression. As previously reported, IL-1 (Interleukin-1) plays a critical role in inflammation and immune cell recruitment during infection. Dysregulation of IL-1 contributed to enhanced illness in *M. tuberculosis* infected mice, making it a targeted for host-directed TB drug strategies (Silvério *et al.* 2021). *MMP12* (macrophage metalloelastase) gene, participate in cell migration, leukocyte activation, antimicrobial defence, and many other crucial processes of immunity and repair (Parasa *et al.* 2017). Similarly, *CXCL5*, aids in immune defence against M. bovis through its involvement in chemokine mediated immune responses. Studies show that lungs of *CXCL5*-deficient mice reconstituted with wild-type bone marrow produced *CXCL5*, suggesting that lung tissue cells and blood-activated platelets release CXCL5 during infection (Nouailles *et al.* 2014). Table 3 lists the top 20 DEGs, with the full list and p-values available in Supplementary table 1.

Table 3.The 20 most highly differentially expressed genes and their *p-values*

Entrez Transcript id	Gene symbol	Fisher value	Stouffer value
KEH36_p11	COX1	6.65E-06	6.00E-05
NM_001002885.1	TMSB4X	8.30E-05	5.36E-04
NM_001033617.2	CTSC	6.62E-06	1.48E-04
NM_001033618.1	ACTG1	5.69E-04	3.40E-04
NM_001206640.1	MMP12	0.00E+00	0.00E+00
NM_173893.3	B2M	3.80E-09	1.06E-05
NM_173925.2	CXCL8	0.00E+00	0.00E+00
NM_173969.3	VIM	3.24E-046	7.29E-04
NM_173979.3	ACTB	1.95E-04	1.60E-04
NM_174062.4	FTH1	9.65E-09	1.69E-06
NM_174093.1	IL1B	0.00E+00	0.00E+00
NM_174187.2	SPP1	1.77E-06	6.07E-04
NM_174300.2	CXCL5	6.73E-12	1.24E-07
NM_174345.4	HSPA8	1.12E-08	1.09E-05
NM_174431.1	PRDX1	2.24E-08	6.39E-06
NM_174744.2	MMP9	2.67E-06	4.41E-05
NM_001077835.1	CTSZ	7.91E-10	1.08E-05
NM_181016.3	SAA3	1.00E-12	1.07E-07
XM_002691119.5	LOC783680	3.40E-09	2.51E-05
XM_005203612.3	S100A12	2.39E-10	6.80E-07

Functional annotation and overrepresentation test: To investigate the functional role of identified DEGS, statistical overrepresentation test for biological processes, molecular functions and cellular components was performed which revealed 1421 significant biological processes, primarily associated with immune responses. Notably, 12 genes were associated with natural killer T-cell activation (GO:0051135; GO:0051133), proliferation (GO:0051142; GO:0051140) and regulation. Bovine natural killer cells have been shown to reduce *M. bovis* BCG in infected macrophages by acquiring cytotoxic/effector activity following activation with IL-12/15 (Endsley *et al.* 2005). Key innate immune response mechanisms included the regulation of MyD88-dependent toll-like receptor signalling pathway (GO:0034124),

TRIF-dependent toll-like receptor signalling pathway (GO:0035666), regulation of interleukin-1-mediated signalling pathway (GO:2000659), regulation of T-helper 1 cell cytokine production (GO:2000554), *NLRP3* inflammasome complex assembly (GO:0044546), etc. These pathways are crucial for recognizing pathogens via PAMPs (pathogen-associated molecular patterns) and initiating immune response. The MyD88 pathway activates pro-inflammatory cytokines, while the TRIF pathway enhances antiviral activity by inducing type I interferons, demonstrating how innate immunity quickly responds to pathogens (Chaplin 2010). Genes such as *IRF1* and *IRF7* mapped to the MyD88-dependent toll-like receptor signalling pathway whereas *IRF3*, *TICAM1*, *TICAM2* and *TLR4* mapped to the TRIF-dependent signalling pathway. NLRP3 inflammasome, a protein complex aids in body's response to infection and cellular damage are activated by PAMPS or damage-associated molecular patterns (DAMPs) (Leu *et al.* 2023) is essential for processing pro-inflammatory cytokines such as IL-1β and IL-18, required for initiation of inflammatory responses, potentiating host defence against pathogens and maintaining immune system homeostasis (Paludan *et al.* 2021). *SIRT2*, *PYCARD* and *NLRP3* genes mapped to the *NLRP3* inflammasome complex assembly. The innate immune system defends against pathogens via these complex network cellular processes and signalling pathways. This system recognizes infections, controls inflammation, and triggers adaptive responses. Understanding these mechanisms is important for developing of therapeutic strategies against infectious agents and inflammatory conditions. Significant molecular functions (237) included Rho-dependent protein serine/threonine kinase activity (GO:0072518), proteasome-activating activity (GO:0036402), nucleobase binding (GO:0002054), histone H4 demethylase activity (GO:0141058), 2'-5'-oligoadenylate synthetase activity (GO:0001730) as the top five with fold enrichment of 5.88. Thirteen out of 224 significant cellular components had a fold enrichment of 5.88 (the highest) which included macrophage migration inhibitory factor receptor complex (GO:0035692), IkappaB kinase complex (GO:0008385), bleb (GO:0032059), DSIF complex (GO:0032044), CKM complex (GO:1990508), etc. Full list of enriched gene ontologies is provided in supplementary table 2.

Overrepresentation test for *Panther* pathways identified 36 significant pathways (Fig. 2), included JAK/STAT signalling pathway, Pentose phosphate pathway, Ubiquitin proteasome pathway, Toll receptor signalling pathway, P53 pathway feedback loops 1, Interleukin signalling pathway, *etc.* as the key pathways. The JAK/STAT signalling pathway critical for inflammation and immune response is often supressed by *M. bovis* through SOCS proteins, enabling immune invasion. (Hasankhani *et al.* 2022). The ubiquitin-proteasome pathway which is responsible for protein degradation and immunological events play a role in BTB by regulating the turnover of proteins associated with apoptosis and inflammation. To enhance its survival within host macrophages, *M. bovis* has the potential to manipulate protein degradation pathways through alterations in host cell signalling cascades. The ubiquitin proteasome

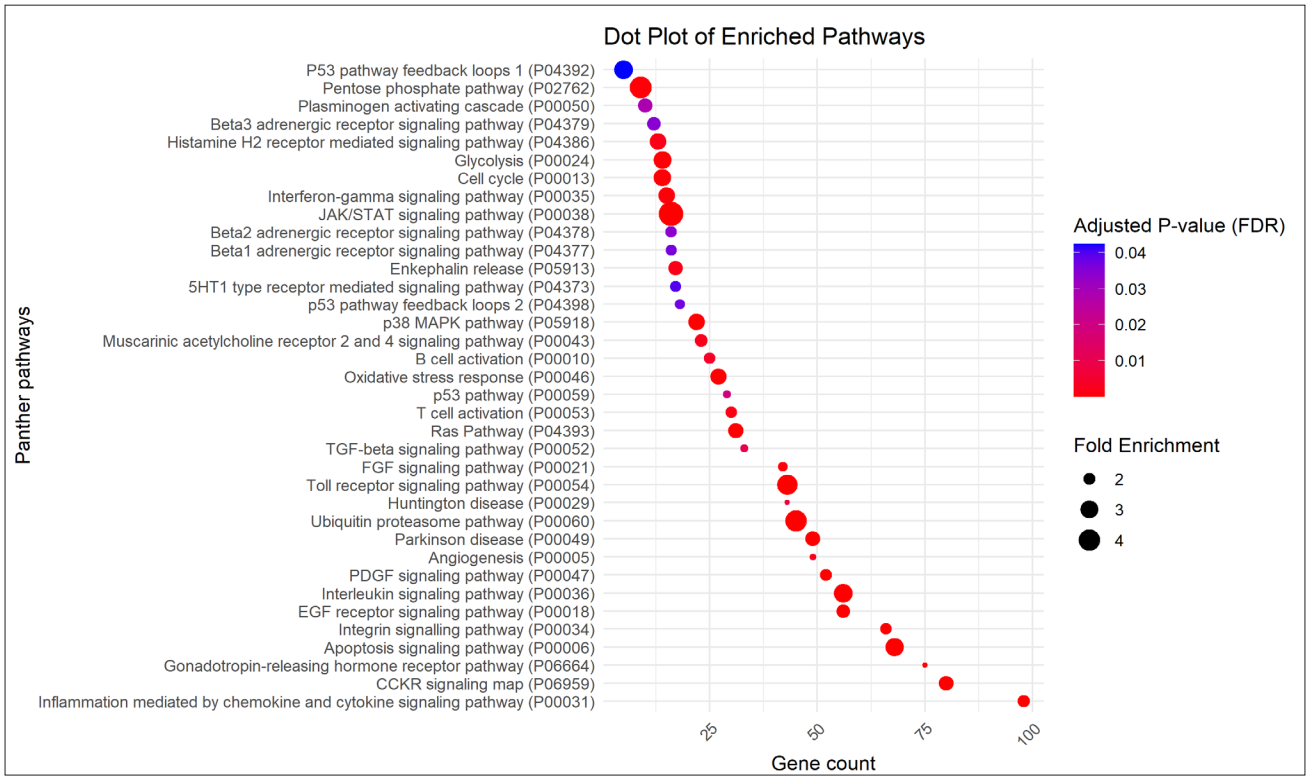

Fig. 2. Dot plot of enriched pathways from functional overrepresentation analysis. Dot size indicates fold enrichment, and colour scale shows adjusted p-values.

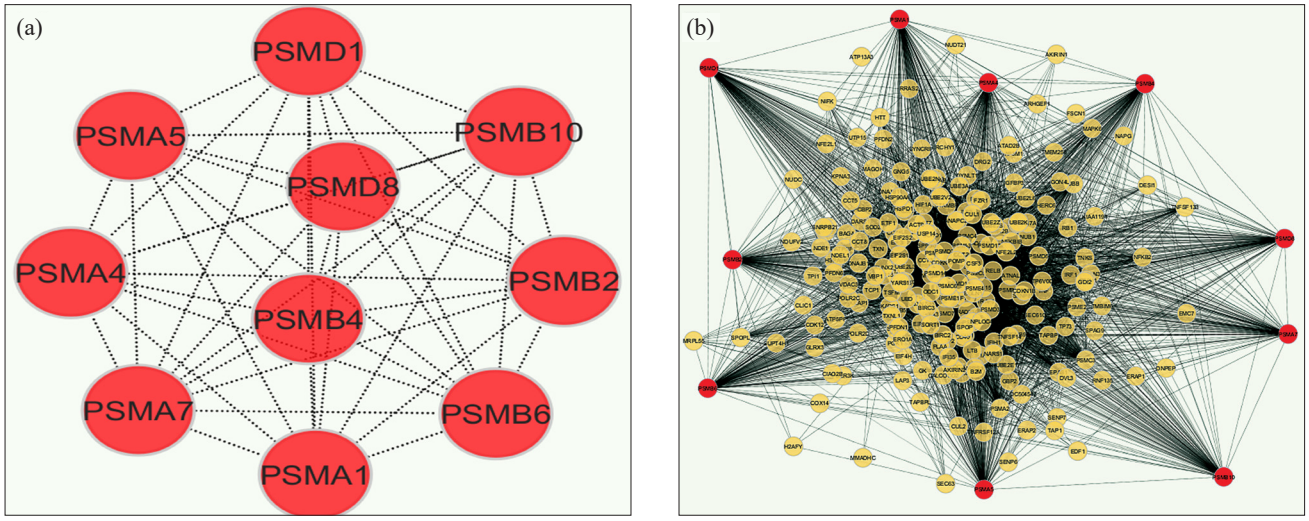

Fig. 3(a) Network of all the ten hub genes identified by the MCC algorithm in PPI network. 3(b) Hub genes and its neighbour proteins in expanded network

pathway mapped to nearly 45 genes in cattle that included genes related to ubiquitin conjugating enzymes (*UBE2V2, UBE2A, UBE2K, UBE2L6, UBE2E1, UBE2L6, UBE2L3*) and proteasome complex subunits *(PSMD8, PSMD11, PSMC6, PSMC4, PSMD7, PSMC1, PSMD13, PSMC3, PSMD6, PSMD4, PSMD14, PSMD2 and PSMD3).*

Hub genes identification from the protein-protein interaction network: The protein-protein interaction network consisted of 3512 nodes and 132474 edges with an average of 37.74 neighbours per node, clustering coefficient of 0.246 and network density of 0.011. The topological analysis revealed *GAPDH* (ENSBTAP00000037577) node has the highest degree (k = 812) that can be considered as a hub (Chen *et al.* 2019) and a bottleneck (2nd highest Betweenness centrality = 0.029) gene. *CDC5L* (ENSBTAP00000026654) emerged as the bottleneck gene with the highest betweenness centrality (BC=0.033) and a degree (k = 712) higher than average. The top 10% genes based on degree is given in supplementary table 3.

GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) beyond its glycolytic role, interacts with the antimicrobial peptide lactoferrin, enhancing the internalization of the peptide by macrophages and promoting autophagy for better intracellular pathogens removal. This process is necessary in reducing the amount of bacterial burden in infected cells (Dhiman *et al.* 2023). Ten hub genes, *namely PSMB3, PSMD8, PSMA5, PSMA1, PSMB2, PSMA4, PSMB10, PSMA7, PSMD1* and *PSMB4* were identified by ranking the genes based on the MCC algorithm that are largely associated with proteasome complex. Hub genes are often considered essential for the stability and function of the overall network and may serve as potential biomarkers or therapeutic targets (Fu *et al.* 2019). The description of each hub gene is given in supplementary table 4. Proteases are highly involved in the breakdown and regulation of proteins, especially in immunological reactions where they degrade antigens to make them presentable via the major histocompatibility complex (MHC) pathway. The immune system uses a proteasome to degrade destructive proteins in diseases such as BTB so that immune cells can construct a defence (Paludan *et al.* 2021). Thus, these genes play a very vital role in regulating the host immune response to *M. bovis*. Genes encoding 20S proteasome subunits (*PSMB3*, *PSMB2*, *PSMB10*, and *PSMB4)* mediate proteolytic degradation of damaged or misfolded proteins while proteasome subunits encoded by *PSMA1*, *PSMA5*, *PSMA4*, and *PSMA7* maintain proteasome structure and function during infection. Previous studies have reported the upregulation of proteasomal complex genes such as *PSMB2*, *PSMB3*, *PSMA2*, *PSMD2*, *PSMD8*, *PSMB6* and *PSMB9* in Mb04-303 (virulent strain of *M. bovis*) infections (Blanco *et al.* 2023). The upregulation of these genes likely indicates an increased need for antigen processing, enhancing the immune system's ability to detect and fight the infection. However, these genes are also linked to the ubiquitin proteasome pathway, which pathogens like *M. bovis* may exploit to degrade host immune cells, similar to the mechanism in *M. tuberculosis* infections in humans. A thorough investigation of this pathway is needed to understand its impact on cattle immunity. Figures 3(a) and 3(b) display a subnetwork of hub-genes and their interactions with neighbouring genes, respectively.

In the present study, 2520 common DEGs were identified in meta-analysis, identifying important immune-related pathways together with important hub genes involved in BTB pathogenesis. Hub genes such as those linked with ubiquitin proteasome pathway, present potential biomarkers and therapeutic targets. Genes elevated during BTB, like CXCL5 and MMP12, may serve as early diagnostic markers of disease progression and treatment efficacy. Future work can be focussed on functional validation of the identified DEGs such as by using CRISPR-Cas9 gene editing methods. This will help in identifying targets for vaccine development for BTB in cattle. Thus, this work offers a comprehensive perspective on the understanding of host-pathogen interactions in the

situation of BTB and opens novel possibilities for future research on diagnostics and therapeutic interventions.

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