Research Article

Effect of *Mycoplasma bovis* on Production of Pro-Inflammatory Cytokines by Peripheral Blood Mononuclear Cells

Rekha Valsala[1], Rajneesh Rana[2*], Arun Thachappully Remesh[1], Sabarinath Thankappan[3], Sabita Behera[1]

[1]Division of Bacteriology & Mycology, IVRI, Izatnagar, Bareilly, UP, India; [2]Division of Bacteriology & Mycology, IVRI, Izatnagar, Bareilly, UP, India; [3]Division of Bacteriology & Mycology, IVRI, Izatnagar, Bareilly, UP, India.

Abstract | *Mycoplasma bovis* is an important bovine pathogen associated with calf pneumonia, mastitis, arthritis and sepicemia. It is known to persist in phagocytic and nonphagocytic cells and disseminate to various organs causing systemic infection. In this study, the effect of live *M. bovis* on pro-inflammatory cytokine production by bovine peripheral blood mononuclear cells was assessed by relative quantification. Temporal change in cytokine levels was assessed at 8 hourly intervals. IL-6 was upregulated (9.32 fold at 24h) by live *M. bovis* at MOI of 1: 10, while TNF-α levels remained unchanged over time. IL-1 and TNF-α levels were not altered by *M. bovis* as compared to the control group. This showed that pro-inflammatory cytokines are not much upregulated at low multiplicity of infection.

Keywords | *Mycoplasma bovis*, IL-1, IL-6, TNF-α, Bovine PBMC

INTRODUCTION

Mycoplasmas are the minimalist microbes, with bare minimum genes as compared to other bacteria and have limited metabolic capability for replication and survival. Even though they are having only half the sets of genes compared to other bacteria, they are pathogenic and are capable of producing variety of diseases in different host species. Besides, many economically important diseases in ruminants like CBPP (Contagious Bovine Pleuropneumonia), CCPP (Contagious Caprine Pleuropneumonia), contagious agalactia, calf pneumonia etc. are caused by mycoplasmas.

Mycoplasma bovis is one of the important agents associated with a number of diseases in bovines, ranging from pneumonia and arthritis in calves to mastitis, genital tract infections, kerato conjunctivitis, otitis media etc. in adult ones (Adamu et al., 2013). Drop in milk production and

calf mortality resulting from the disease produces huge economic impact to the dairy industry. The ability of the organism to get adapted to various tissues and to produce diseases indicates the diversity of still unclear pathogenic mechanisms. In many infections, *M. bovis* acts as an exacerbating agent and often goes undiagnosed or overlooked.

Very little knowledge is available regarding the pathogenic mechanism and virulence factors contributing to mycoplasmal diseases in spite of the small sized genome with limited coding capacity. First step in pathogenesis is adhesion and colonisation (Razin and Jacobs, 1992). Certain adhesins have been identified in mycoplasmas. Mycoplasmal membrane is important in colonization and establishment of infection, as the organism lacks cell wall (Rottem, 2003). Other pathogenic mechanisms include production of hydrogen peroxide and superoxide radicals (Schott et al., 2014). Moreover, membrane lipoproteins, which undergo phase and size variation aid in immune

evasion (Rosengarten et al., 2000). Phase and size variation of membrane lipoproteins aid in evading the host immune response (Behrens et al., 1994). A lymphoinhibitory peptide has been identified in *M. bovis* (Vanden Bush and Rosenbusch, 2004). *M. bovis* is able to invade and persist in phagocytic as well as non-phagocytic cells, which allows systemic spread of infection without being recognised by host immune system (Burki et al., 2015).

Immune response contributes to the pulmonary tissue damage characteristic of mycoplasmal respiratory infections (Van den Bush, 2003). Interaction of mycoplasma with host immune cells results in production of pro-inflammatory cytokines as well as chemokines. The production of nitric oxide and TNF-α are induced in alveolar macrophages on co-incubation with *M. bovis* (Jungi et al., 1996). Besides bovine γδ T-cells are activated, which are also part of innate immunity. Van der Merwe et al. (2010) showed persistence of *M. bovis* in bovine peripheral blood mononuclear cells and erythrocytes. This study was conducted to evaluate the effect of *M. bovis* on production of pro-inflammatory cytokines by bovine PBMCs.

RESULTS AND DISCUSSION

PBMCs were isolated from cattle blood by density gradient centrifugation. The cell count in bovine PBMC was 1-1.225 x 10^6 cells/ml and viability percentage was 91.49-93.02%. RNA was extracted from PBMC samples after co-incubation for different time periods with live *M. bovis.* On analysis of purity and concentration of RNA samples by nanodrop, all the samples were having A260/A280 between 1.8-2.0, indicating purity and concentration ranged from 500-1000 ng/μl. cDNA was synthesized from all the RNA samples using Revert Aid First Strand cDNA synthesis kit.

The relative expression of IL-1, IL-6 and TNF-α mRNA were calculated in terms of fold change with respect to housekeeping gene YWHAZ. YWHAZ codes for Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide and was earlier reported to be a suitable reference gene for bovine samples (Spalenza et al., 2011). Previously reported primers which bind at the exon-exon junctions were used in this study (Yamada et al., 2009; Puech et al., 2015). Group I represents bovine PBMC treated with live *M. bovis* at MOI of 100:1 and group II represents untreated control. Results are represented in Figure 1, 2 and 3 respectively for IL-1, IL-6 and TNF-α.IL-1 expression level was similar in both groups at 8h, followed by a significant upregulation at 16 h. At 24h, the IL-1 expression level reduced in both the groups, but the level was lower in group treated with *M. bovis*. This result indicated that *M. bovis* co-infection resulted in a comparatively lower IL-1 response. Reduced IL-1 mRNA
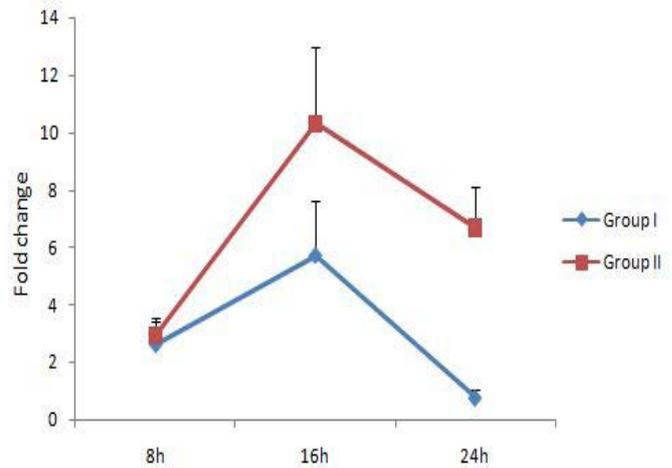

Figure 1: Graph depicting relative change in IL-1 expression over time

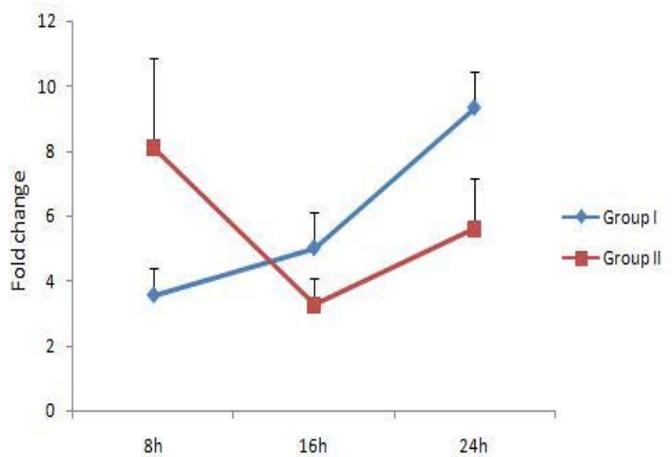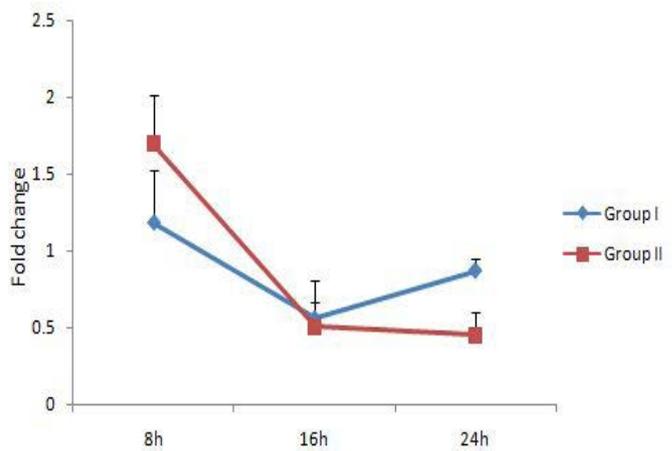**Figure 2:** Graph depicting relative change in IL-6 expression over time

Figure 3: Graph depicting relative change in TNF-α expression over time

expression level has been demonstrated previously in squamous cell carcinoma A431 cells infected with mycoplasmas as compared to non-infected cells (Demczuk et al., 1988). IL-6 gene expression level was showing an increasing trend over time and was significantly upregulat-

ed in group I (9.32 fold) at 24h. But the expression level reduced in group II at 16h as compared to 8h followed by an increase at 24h.TNF-α level was not showing any significant changes with time. These results showed that *M. bovis*co-infection resulted in upregulation of the pro-inflammatory cytokine IL-6 alone and a comparatively lower IL-1 response.

Inflammatory cytokines are part of body's immediate defense response to 'danger' and immune response is greatly enhanced by them (Murtaugh and Foss, 2002). They may play an important role in enhancing the biological response of bovine neutrophils to bacterial infections (Leite et al., 2002; Sohn et al., 2007). Rodriguez et al. (2015) conducted immunohisto chemical study for cytokine expression in spontaneously infected animals and found out that *M. bovis*-associated pneumonic lesions consistently resulted in upregulation of TNF-α, IL-4, IL-10 and IFN-γ expression, but expression of IL-1α, IL-1β, IL-2, IL-6 and IL-8 did not exhibit significant differences between infected and normal lung from control calves. In a similar study by Gondaira et al. (2015) live *M. bovis* was shown to significantly induce the production of tumor necrosis factor alpha (TNF-α), interleukin 12p40 (IL-12), and interferon gamma (IFN-γ) mRNA expression in bovine PBMCs at a multiplicity of infection (MOI) of 1000 but not at MOI of 10 and 100. The relatively lower inflammatory cytokine response in our study might be due to the low MOI.

Alveolar macrophages are activated by *M. bovis* and produce TNF-α and nitric oxide (Jungi et al., 1996).This is important in pathogenesis as well. Jimbo et al. (2017) showed *in vitro* production of TNF-α and IL-12 by neutrophils on infection with *M. bovis* priming to Th1 response. In monocytes, *M. bovis* delays apoptosis, and suppresses production of IFN-γ and TNF-α (Mulongo et al., 2014), which aids in persistence and systemic dissemination of *M. bovis* by effective immunomodulation.

Production of pro-inflammatory cytokines is important with respect to pathogenesis of *M. bovis*. Although there was an upregulation of IL-6 on co-incubation of PBMCs with live *M. bovis* at an MOI of 100:1, the levels of IL-1 and TNF- α were not increased. It can be concluded that it is required to have high multiplicity of infection for producing a consistent inflammatory response.

MATERIALS AND METHODS

Mycoplasma bovis Standard strain NCTC 10131 was obtained from Referral Laboratory on Mycoplasma, Division of Bacteriology and Mycology, Indian Veterinary Research Institute, Izatnagar (U.P) and was grown in modified PPLO broth medium (PPLO broth, yeast extract, glucose,

sodium pyruvate, phenol red 0.5%, thallous acetate 5%, penicillin, DNA 0.2%, horse serum 10%). Pelleted the cells by refrigerated centrifugation at 10000xg for 15 min. After one was step with phosphate buffered saline (PBS, pH 7.4) *M. bovis* was resuspended in PBS. The concentration was adjusted to approximately 10^8 cells/ml.

ISOLATION OF BOVINE PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMCS)
Blood (10 ml) was collected in anticoagulant ACD-A containing tubes from 3 healthy animals and was used for PBMC isolation by gradient centrifugation over histo-paque 1083 (Sigma Aldrich) following the manufacturer's protocol. PBMCs were resuspended in RPMI-1640 medium containing horse serum and antibiotics. Viable cell count was determined by counting in haemocytometer counting chamber following trypan blue staining.

CO-INCUBATION WITH LIVE *M. BOVIS*
PBMC concentration was adjusted to 2 x 10^6 cells/ ml and 100 µl each was added to wells in a 96 well tissue culture plate. In one row, *M. bovis* was added at an MOI of100:1. Cells from each animal were plated in triplicate. The cells were incubated in 3 different plates at 37^0C, 5% CO_2. One plate each was removed at 8h intervals.

TOTAL RNA EXTRACTION
Total RNA extraction from the PBMC samples collected was done by Ribozol method. The concentration of RNA was checked by Nanodrop (NanoVue Plus, GE Lifesciences). RNA purity was determined by 260:280 ratio. Total RNA with the ratio values 1.8 or greater are deemed acceptable for subsequent procedure and was stored at -70ºC.

REVERSE TRANSCRIPTION FOR CDNA SYNTHESIS
Synthesis of cDNA from total RNA was carried out by using Revert Aid First Strand cDNA synthesis kit (Thermoscientific, U.S.A.). In a nuclease free tube 1000 ng of total RNA was taken and to this 1µl of oligo(dT) primer (100µM) was added. Total volume was adjusted to 12 µl using nuclease free water. The contents were gently mixed and incubated at 65ºC for 5 minutes. The tube was then chilled on ice followed by addition of 4µl 5X reaction buffer, 1 µl of RiboLock RNase Inhibitor (20 U/µl), 2 µl of 10 mM dNTP Mix, and 1 µl of RevertAid M-MuLV Reverse Transcriptase (200 U/µl) to get a final volume of 20 µl. The reaction mixture was mixed and centrifuged briefly and then incubated at 42ºC for 60 minutes. The reaction was terminated by heating at 70ºC for 5 minutes. Finally the product was stored at -20ºC.

PRO-INFLAMMATORY CYTOKINE EXPRESSION STUDY BY REAL TIME PCR
Study of differential expression of 3 cytokine genes IL-1, 1L-6 and TNF-αm RNA from PBMC samples was done

by relative quantification. Real-time PCR was carried out using YWHAZ as reference housekeeping gene (endogenous control). Primers used for real time PCR are given in Table 1. All the primers were procured from Eurofins MWG Operon, Bangalore. Reaction was carried out in a total volume of 15μl. For each sample, a non-template control (NTC) for both target and endogenous genes, which included all ingredients of real time reaction except the template, was run to check contamination of any ingredient.

Table 1: Real time PCR primers

Gene	Primer (5'-3')
IL-1	CCTTGGGTATCAGGGACAA
	GGGTATGGCTTTCTTTAGG
IL-6	TGAGTGTGAAAGCAGCAAGGA
	TACTCCAGAAGACCAGCAGTGG
TNF-α	CCAGAGGGAAGAGCAGTCC
	GGCTACAACGTGGGCTACC
YWHAZ	GAAAGGGATTGTGGACCAG
	GGCTTCATCAAATGCTGTCT

Real-time PCR was performed on a CFX96 C1000 Touch QPCR System (Biorad, U.S.A.) using Kapa SYBR Green mastermix (Sigma Aldrich, U.S.A.). Samples were run in duplicates and relative expression was studied. Three stage PCR protocol was set for the real-time amplification of cDNA for each and every sample. First stage consisted of initial melting of template for 3 minutes at 95°C with no repetition. Second stage consisted of 2 steps repeated forty times with first step of melting template at 95°C for 10 seconds and second step of annealing/extension at 60°C for 30 seconds except for IL-6. For IL-6, annealing was at 54°C for 15sec followed by an extension step at 72°C for15 sec. Third stage consisted of three steps and is carried out for drawing the dissociation curve of products generated during second stage.

After 40 cycles of amplification, threshold cycle (Ct) value was obtained for PBMC cytokines and internal control gene YWHAZ (reference gene). Melting curve analysis was performed for each sample to verify the specificity of each product. The fluorescent data acquisition was continuous from annealing to the final denaturation step. The Real time data obtained was analyzed by CFX96 Touch QPCR Software (Biorad, U.S.A.). Ct values were used to determine the relative quantification of cytokines.

The change in the level of expression of PBMC cytokine mRNA was calculated by using the ΔΔCt method. The

statistical significance of differences in mRNA expressions of the examined factors was assessed by one way ANOVA test using SPSS 20.0 software. Differences were considered significant if $P<0.05$.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interests.

AUTHORS CONTRIBUTION

All the authors have contributed in terms of technical knowledge in framing the article.

REFERENCES

- Adamu JY, Wawegama NK, Browning GF, Markham PF (2013). Membrane proteins of *Mycoplasma bovis* and their role in pathogenesis. Res. Vet. Sci. 95: 321-325. https://doi.org/10.1016/j.rvsc.2013.05.016
- Behrens A, Heller M, Kirchoff H, Yogev D, Rosengarten R (1994). A family of phase- and size- variant membrane surface lipoprotein antigens (Vsps) of *Mycoplasma bovis*. Infect. Immun. 62: 5075-5084.
- Burki S, Fey J, Pilo P (2015). Virulence, persistence and dissemination of *Mycoplasma bovis*. Vet. Microbiol. http://dx.doi.org/10.1016/j.vetmic.2015.02.024.
- Demczuk S, Baumberger C, Macho B, Dayer JM (1988). Differential effects of *in vitro* Mycoplasma infection on Interleukin-1α and β mRNA expression in U937 and A431 cells. J. Biol. Chem. 263: 13039-13045.
- Gondaira S, Higuchi H, Iwano H, Nakajima K, Kawai K, Hashiguchi S,Konnai S, Nagahota H (2015). Cytokine mRNA profiling and the proliferative response of bovine peripheral blood mononuclear cells to *Mycoplasma bovis*. Vet. Immunol. Immunopathol. 165: 45-53. https://doi.org/10.1016/j.vetimm.2015.03.002
- Jimbo S, Sulehman M, Maina T, Prysliak T, Mulongo M, Perez-Casal J (2017). Effect of *Mycoplasma bovis* on bovine neutrophils. Vet. Immunol. Immunopath. 188: 27-33. https://doi.org/10.1016/j.vetimm.2017.04.011
- Jungi TW, Krampe M, Sileghem M, GriotCh, Nicolet J (1996). Differential and strain-specific triggering of bovine alveolar macrophage effector functions by mycoplasmas . Microbial Path. 21: 487-498. https://doi.org/10.1006/mpat.1996.0078
- Leite F, O'Brien S, Sylte MJ, Page T, Atapattu D, Czuprynski CJ(2002). Inflammatory cytokines enhance the interaction of *Mannheimia haemolytica* leukotoxin with bovine peripheral

blood neutrophils *in vitro*. Infect. Immun. 70: 4336–4343. https://doi.org/10.1128/IAI.70.8.4336-4343.2002

• Mulongo M, Prysliak T, Scruten E, Napper S, Perez-casal J (2014). *In vitro* infection of bovine monocytes with *Mycoplasma bovis* delays apoptosis and suppresses production of Gamma Interferon and Tumor Necrosis Factor Alpha but not Interleukin-10. Infect. Immun. 82: 62-71. https://doi.org/10.1128/IAI.00961-13

• Murtaugh M P, Foss DL (2002). Inflammatory cytokines and antigen presenting cell activation. Vet. Immunol. Immunopathol. 87: 109–121. https://doi.org/10.1016/S0165-2427(02)00042-9

• Puech C, Dedieu L, Chantal I, Rodrigues V (2015). Design and evaluation of a unique SYBR Green real-time RT-PCR assay for quantification of five major cytokine genes in cattle, sheep and goat. BMC Vet. Res. https://doi.org/10.1186/s12917-015-0382-0

• Razin S, Jacobs E (1992). Mycoplasma adhesion. J. Gen. Microbiol. 138: 407-422. https://doi.org/10.1099/00221287-138-3-407

• Rodriguez F, Castro P, Poveda JB, Afonso AM, Fernandez A (2015). Immunohisto chemical labelling of cytokines in calves infected experimentally with *Mycoplasma bovis*. J. Comp. Pathol. 152: 243-247. https://doi.org/10.1016/j.jcpa.2015.01.006

• Rosengarten R, Citti C, Glew M, Lischewski A, Droebe M, Much P, Winner F, Brank M, Spergser J (2000). Host-pathogen interactions in mycoplasma pathogenesis: Virulence and survival strategies of minimalist prokaryotes. Int. J. Med. Microbiol. 290: 15-25. https://doi.org/10.1016/S1438-4221(00)80099-5

• Rottem S (2003). Interaction of Mycoplasmas with host cells. Physiol. Rev. S3: 417-432. https://doi.org/10.1152/physrev.00030.2002

• Schott C, Cai H, Parker L, Bateman KG, Caswell JL (2014). Hydrogen peroxide production and free radical-mediated cell stress in *Mycoplasma bovis* pneumonia. J. Comp. Pathol. 150: 127-137. https://doi.org/10.1016/j.jcpa.2013.07.008

• Sohn EJ, Paape MJ, Connor EE, Bannerman DD, Fetterer RH, Peters RR (2007). Bacterial lipopolysaccharide stimulates bovine neutrophil production of TNF-alpha, IL-1beta, IL-12 and IFN-gamma. Vet. Res. 38: 809–818. https://doi.org/10.1051/vetres:2007033

• Spalenza V, Girolami F, Bevilacqua C, Riondato F, Rasero R, Nebbia C, Sacchi P, Martin P (2011). Identification of internal control genes for quantitative expression analysis by real-time PCR in bovine peripheral lymphocytes. Vet. J. 189: 278–283. https://doi.org/10.1016/j.tvjl.2010.11.017

• Van der Merwe J, Prysliak T, Perez-Casal J (2010). Invasion of bovine peripheral blood mononuclear cells and erythrocytes by *Mycoplasma bovis*. Infect. Immun. 78: 4570–4578. https://doi.org/10.1128/IAI.00707-10

• Vanden Bush TJ, Rosenbusch RF (2003). Characterization of the immune response to *Mycoplasma bovis* lung infection. Vet. Immunol. Immunopathol. 94: 23-33. https://doi.org/10.1016/S0165-2427(03)00056-4.

• Vanden Bush TJ, Rosenbusch RF (2004). Characterization of a lympho-inhibitory peptide produced by *Mycoplasma bovis*. Biochem. Biophys. Res. Commun. 315: 336-341. https://doi.org/10.1016/j.bbrc.2004.01.063

• Yamada S, Onnai S, Imamura S, Simuunza M, Chembensofu M, Chota M, Nambota A, Onuma M, Ohashi K (2009). Quantitative Analysis of Cytokine mRNA Expression and Protozoan DNA Load in *Theileria parva*-Infected Cattle. J. Vet. Med. Sci. 71: 49–54. https://doi.org/10.1292/jvms.71.49