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Cytokine gene expression and pathology in experimental *Pasteurella multocida* infection in mice

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ABSTRACT

The study was conducted to know the pathology and cytokine gene expression studies in experimental infection with *Pasteurella multocida* in mice. Swiss albino mice (20) were inoculated with *P. multocida* (200 µl of 1×10^8 cfu/ml) and control mice with sterile PBS intraperitoneally and sacrificed 4 mice at 6, 12, 18 and 24 h. Mice showed dullness, lethargy and unable to move at 18 and 24 h after inoculation. Histopathology revealed changes in liver, kidney, spleen, lung and no observable changes in heart and abdominal muscles. Liver showed septicemia condition and acute bronchopneumonia of lung at 24 h. TNF-α gene showed 20-fold increase in liver and spleen and 5-folds in kidney. There was upregulation of pro-inflammatory cytokines in liver, kidney, spleen and IL–10 gene at later periods of infection which is a new finding, needs further study. Thus, the present study indicated that increase in tissue cytokine gene expression concurred with histopathological changes were attributed to the pathogenesis of Pasteurellosis in mice.

Key words: Cytokine gene expression, Mice, *Pasteurella multocida*, Pathology

Pasteurella (P.) multocida, a gram negative bacteria causing various diseases in livestock and birds, occurs as commensal pathogen in upper respiratory tract and causes respiratory diseases in bovines resulting in severe mortality especially in buffaloes. The bacterial components such as capsular or outer membrane proteins and endotoxins have been reported as virulence factors responsible for immunopathological changes (Boyce and Adler 2006). The immune system is involved in infection control and activates different cellular mechanisms, in which cytokines play an important role. Cytokines are low molecular weight proteins which act as intercellular mediators involved in many biological processes such as inflammation, fibrosis, angiogenesis, cell growth, cell proliferation and immune response (Tizard 2008). However, the mechanism of acute disease pathogenesis and role of bacterial or host factors that induce pathology are poorly understood. Serum cytokine profiles showed significantly higher amount of proinflammatory cytokines like Tumour necrosis factor-alpha (TNF-α), Interleukin 1-beta (IL-1β), IL-6 and mouse KC in the *P. multocida* serotype A1 infected mice when compared to the control mice (Praveena *et al.* 2010). However, there is no data regarding the cytokine gene expression studies in *P. multocida* infection such as IL-2, IL-4, TNF-α and anti-inflammatory cytokine IL-10, in liver, kidney and spleen of mice. Hence, the present study was undertaken on the pathology and cytokine gene expression in liver, kidney, spleen of mice infected with *P. multocida* at different time points.

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MATERIALS AND METHODS

Male Swiss albino mice (20), 6–8 weeks of age weighing 25–30 g, were procured from National Centre for Laboratory Animal Sciences, Hyderabad. Mice were housed in individually ventilated cages and fed with pellet rodent feed and purified water *ad lib*. The animal experiments was approved by Institutional Animal Ethics Committee of ICAR-NIVEDI, Bengaluru (CPCSEA Registration No. 881/R/S/05/CPCSEA) and performed as per Committee for the Purpose of Control and Supervision of Experiments on Animal (CPCSEA) guidelines, New Delhi. The American Type Culture Collection *Pasteurella multocida* subsp. *multocida* (ATCC® 12945™) was used for inoculation in to the Swiss albino mice. Mice were divided into five groups of four mice each and four groups were given *P. multocida* inoculum of 200 µl containing 1×10^8 CFU/ml (Kapoor *et al.* 2004, Praveena *et al.* 2010) and control group with sterile PBS by intraperitoneal route. The mice were observed for clinical signs and four mice were sacrificed at 6, 12, 18 and 24 h intervals by using overdose of Ketamine hydrochloride.

Blood was collected from intracardiac route, smear prepared on grease free glass slide and stained with Gram's stain. The stained slides were examined under light microscopy and observed for the presence of *P. multocida* in oil immersion field. Blood was collected in potassium EDTA coated vacutainer tubes and genomic DNA was

isolated using QIAmp DNA mini kit (Qiagen, USA) as per kit manufacturer's instructions. Using *P. multocida* species specific primers, the purified DNA was amplified using thermal cycler (M/s. Eppendropff Master cycler, USA) as described earlier (Townsend *et al.* 1998). At the time of sacrifice, necropsy and gross examination of visceral organs was performed. The liver, kidney, spleen, heart, lung and abdominal muscles were collected in 10% buffered formalin. The formalin fixed tissues were processed, embedded in paraffin, 5 µm tissue sections were prepared using rotatory microtome and stained with haematoxylin and eosin (Bancroft and Stevens 1996) and were examined under light microscope for histological changes.

Mice liver, kidney and spleen tissues were collected in RNA later® solution (Ambion, USA) and stored in deep freezer at –80ºC until use. The RNA extraction was carried out from liver, kidney and spleen tissues by using RNeasy mini kit (Qiagen, USA) and cDNA was synthesised by using Revert aid H-minus first strand cDNA synthesis kit (Fermentas, Thermo Fischer, USA). The cytokine gene expression study was carried in the Real time PCR by using Light cycler® 480 II (Roche Products India Private Limited, Mumbai) by Taqman Probe method. The primers and probes were designed as reported earlier (Krishnamoorthy *et al.* 2016). The details of primers and probe used for various cytokine genes is given in Table 1. The fold change in expression of inflammatory cytokine genes was carried out by using $2^{-\Delta\Delta CT}$ method as described previously (Livak and Schmittgen 2001). The data were obtained by calculating advanced relative quantification method by using Light Cycler® 480 Software release 1.5.0 SP3 version 1.5.0.39 (Roche Products India Private Limited, Mumbai). Data obtained were analyzed using Statistical Analysis System (SAS) software Enterprise Guide version 5.1 (SAS India limited, Mumbai) by using one way analysis of variance (ANOVA) method (Snedecor and Cochran 2012) and obtained the significant difference between different time points. The results were expressed as the mean ± SE (standard error) with significant difference at $P<0.05$ and confidence interval at 95% level.

RESULTS AND DISCUSSIONS

The infected mice were dull and anorectic at 12 h, and had mild crusting around eyes and nose, ruffled hairs and were showing lethargic appearance at 18 h after inoculation of *P. multocida*. At 24 h, mice were depressed, weak and huddled together, unable to move, when compared to control mice which were clinically normal. In the present study, the clinical signs in mice were evident at 18 and 24 h after infection and concurred with previous reports (Praveena *et al.* 2010, Rezaie *et al.* 2014). Blood smear examination could not reveal the presence of bacteria at 6, 12 and 18 h except at 24 h after mice inoculation. PCR with DNA isolated from mice blood revealed presence of *P. multocida* in blood at 18 and 24 h (Fig.1) At 24 h after inoculation, blood smear showed presence of bipolar organisms and confirmed the occurrence of septicemia at later stages of acute infection, which corroborated with previous reports (Praveena *et al.* 2010). This also indicated that PCR is more sensitive than the blood smear examination for *P. multocida* infection in mice.

Gross examination of organs revealed no observable changes however lungs were congested and haemorrhagic in mice sacrificed at 18 and 24 h after infection and concurred with previous reports (Kapoor *et al.* 2004, Praveena *et al*. 2010). Histopathological changes observed

Table 1. Primers used and amplicon size of various cytokine genes in mice

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')	Probe no. & Catalogue No (Universal Probe Library)	Amplicon size
IL 2	GCTGTTGATGGACCTACAGGA	ATCCTGGGGAGTTTCAGGTT	No. 15 (Cat. no. 04685148001)	69
IL 4	CATCGGCATTTTGAACGAG	GACGTTTGGCACATCCATCT	No. 92 (Cat. no. 04684982001)	67
IL 10	ACTCTGTTGCCTGGTCTTCC	GACAGGGTGCTCGCATCT	No. 41 (Cat. no. 04688007001)	60
TNF-α	AAGGGGGACCAACTCAGC	CGGACTCCGCAAAGTCTAAG	No. 113 (Cat. no. 04693477001)	61
GAPDH	AGCTTGTCATCAACGGGAAG	TTTGATGTTAGTGGGGTCTCG	No. 133 (Cat. no. 04685075001)	62

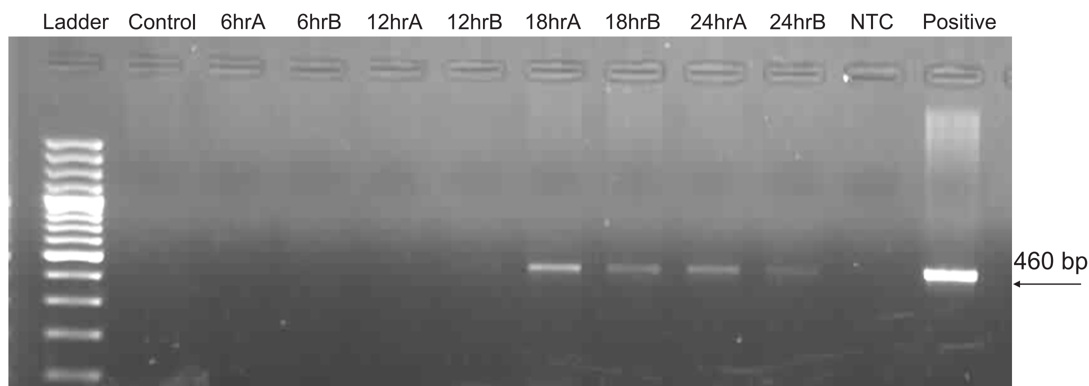

Fig.1. PCR detection of *P. multocida* DNA in mice blood using species specific PMT PCR at different time points.

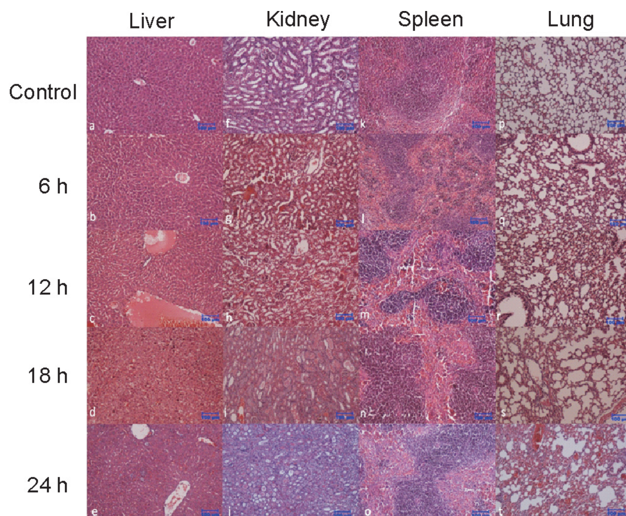

Fig. 2. Photomicrograph showing histopathology of liver, kidney, spleen and lung of control group and mice infected with *P. multocida* at different time points. Liver, kidney, lung showing histological changes due to infection. Liver showing severe changes with bacterial clumps and lung revealed severe infiltration of mononuclear cells with congestion of blood vessels (inset). Haematoxylin and Eosin stain, Scale bar = 100 μm.

in various organs are shown in Fig. 2. Microscopically, liver showed congestion of the blood vessels at 12 h (Fig. 2c) and degenerative changes such as vacuolations in the cytoplasm, swollen nucleus, increased sinusoidal spaces (Fig. 2d) and focal infiltration of polymorphonuclear cells at 18 h (Fig. 2d). At 24 h, micro vesicles containing bacterial organisms was observed (Fig. 2e) along with degenerative changes of hepatocytes and infiltration of inflammatory cells both polymorphonuclear and mononuclear cells with loss of hepatic architecture indicating the damage to the liver parenchyma when compared to control group and at 6 h (Figs 2 a,b). Formation of bacterial organisms in liver indicated the septicaemia caused by *P. multocida* in mice. Kidney showed normal glomerulus and tubular epithelial cells in the control group (Fig. 2f). At 6 h, damage to glomerular architecture and tubular epithelial cells (Fig. 2g) and at 12 h, dilatation of tubules with congestion of blood vessels were observed (Fig. 2h). During 18 and 24 h, the tubules were dilated with accumulation of edema fluid, congestion of blood vessels and loss of glomerular architecture (Figs 2i,j) which indicated the severe damage to kidneys of mice. Spleen of control mice showed the normal white and red pulp architecture (Figs 2k). Spleen of infected mice revealed mild loss of lymphocytes, which progressed from mild to moderate from 6 to 24 h, which was evident by increase in the reticulum fibres (Figs 2l,m,n,o). Lung revealed infiltration of polymorphonuclear cells in the alveolar space and engorged blood vessels after 6 h of infection (Fig. 2q) when compared to control mice with normal alveolar architecture (Fig. 2p). Bronchial lumen showed presence of cellular exudates and interstitial thickening, inflammatory cell infiltration at 12 h after infection (Fig. 2r). At 18 h, there was dilatation of alveoli by coalescence of adjacent alveoli, desquamation of bronchial epithelium (Fig. 2s) and severe infiltration of inflammatory cells mainly polymorphonuclear cells and mononuclear cells with congestion of blood cells (Fig. 2t). Heart and abdominal muscles did not reveal any histological change at all the time points. However, the endocardium of heart showed the presence of bacterial organism attaching to the endocardium wall at 24 h (Figure not shown). Histopathological changes observed in lung and liver concurred with previous reports (Kapoor *et al.* 2004, Praveena *et al.* 2010, Pors *et al*. 2011, Rezaie *et al*. 2014). The bacterial clumps in the liver observed in this study was in agreement with PCR detection of bacterial DNA in blood and also with the previous study (Kapoor *et al*. 2004). Histopathological changes observed in kidney and spleen this study were not reported earlier, which might be due to the change in the pathogenicity of bacteria.

Cytokine gene expression in mice at different time points after infection with *P. multocida* is shown in Fig. 3. The histological changes in tissues suggested that there was infiltration of inflammatory cells which could be triggered by a surge in the pro-inflammatory cytokines like IL-1β, TNF-α, IL-2 and IL-4, in response to cell wall components of Gram-negative bacteria (Locksley *et al.* 2001). In the present study, IL-2 gene expression showed non-significant up-regulation of ten and seven folds in liver and spleen, respectively at 12 h and nearly 2-fold significant ($P<0.05$) increase in kidney at 18 h after infection. IL-4 expression revealed 4-fold increase in liver at 12 h, 2- and 14-fold

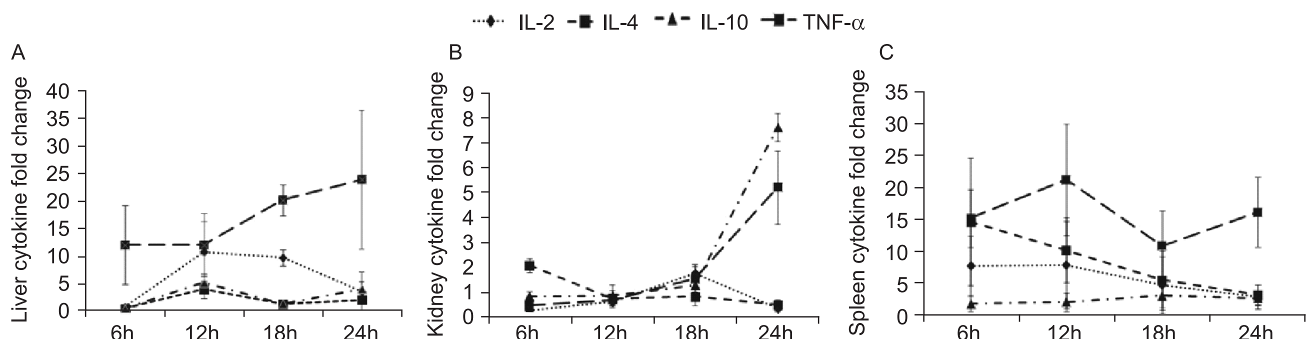

Fig. 3. Cytokine gene expression fold changes in liver (A), kidney (B) and spleen (C) at different time points in mice after *Pasteurella multocida* infection.

increase in kidney and spleen, respectively at 6 h. TNF-α gene expression showed twenty folds increase in liver at 24 h and spleen at 12 h and 5-fold significant ($P<0.05$) increase in kidney at 12 h after infection over and above the control mice. Significantly higher levels of serum TNF-α in *P. multocida* serotype A infected mice at 24 and 48 h was reported previously (Rezaie *et al.* 2014) and concurred with the present study. This suggested that TNF-α plays an important role in mediating the inflammatory processes in the *P. multocida* infection when compared to other cytokine genes. The LPS and porin proteins isolated from *P. multocida* have been reported to upregulate the mRNA expression levels of pro-inflammatory cytokines in murine splenic lymphocytes (Iovane *et al.* 1998). These cytokines may help in migration of leukocyte to the site of infection by favouring the expression of cell adhesion molecules and production of eicosanoids and prostaglandins that leads to the inflammatory cascade in to septicemia. The higher levels of fold changes in the proinflammatory cytokines in liver, kidney and spleen could also be attributed to the fever, anorexia and behavioural changes observed in the infected animals (Locksley *et al.* 2001). IL-10 is a cytokine with multiple, pleiotropic, effects in immunoregulation and inflammation, and considered as an anti-inflammatory cytokine. It downregulates the expression of Th1 cytokines, MHC class II antigens, and co-stimulatory molecules on macrophages. It also enhances B cell survival, proliferation and antibody production. In the present study, IL-10 gene expression showed significant ($P<0.05$) increase of 5-fold at 12 h in liver and seven folds at 24 h in kidney, however a nonsignificant increase of 3-fold was observed in spleen of mice at 18 h. This indicated the important role of anti-inflammatory cytokine IL-10 in *P. multocida* infection and might be mediated by the other cytokines and also due to tissue damage, which is new finding from this study. IL-10 can block NF-κB activity, and is involved in the regulation of the JAK-STAT signalling pathway. The role of anti-inflammatory cytokine IL-10 in *P. multocida* infection in mice was not studied previously and may be stimulated by host tissue factors to control the excessive proinflammatory cytokine gene expression as a feedback response. The role of IL-10 in the bacterial pathogenesis needs further study to explore the possible role in limiting the bacterial infection.

In conclusion, the intraperitoneal infection of *P. multocida* could be better detected by PCR of blood and induced histopathological lesions in liver, kidney, spleen and lung of mice. The cytokine gene expression revealed upregulation of proinflammatory and anti-inflammatory cytokine genes at different time points after infection. Further, increased fold changes were observed in TNF-α in liver, kidney and spleen than IL-2, IL-4 cytokine genes indicated the importance in *P. multocida* infections. The role of IL-10 anti-inflammatory cytokine gene in *P. multocida* infection is a new finding from this study. However, the molecular mechanisms by which the subcellular changes induced by bacterial components has to be studied in detail. The increased expression of both proinflammatory and anti-inflammatory cytokine genes and histological changes in tissues were attributed to pathogenesis of Pasteurellosis and death in mice.

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