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Pro-Inflammatory Cytokine (IL-1β and IL-6) Response in Goats Challenged with *Corynebacterium pseudotuberculosis* and its Immunogen Mycolic Acids

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Abstract: The causative agent of Caseous Lymphadenitis (CLA) is *Corynebacterium pseudotuberculosis*. A chronic disease of sheep and goats which is mainly characterized by suppurative abscesses in both superficial and visceral lymph nodes. There is paucity of information on the proinflammatory cytokine (IL-1β and IL-6) responses due to *C. pseudotuberculosis* and Mycolic Acid (MAs) inoculation in goats. Therefore, this study was conducted to evaluate the immunogenicity of *C. pseudotuberculosis* and MAs. Twelve clinically healthy crossbred Boer female goats were divided into 3 groups; A, B and C with 4 goats per group. The A group was injected with 2 mL sterile Phosphate Buffered Saline (PBS) intradermally as negative control group while B group was inoculated with 2 mL of MAs extract intradermally. Group C was inoculated with 2 mL 1×10^9 colony forming unit (cfu) of live *C. pseudotuberculosis* intradermally. Blood was collected from the jugular vein periodically for IL-1β and IL-6 analysis throughout the experimental period. The results showed significant increase ($p<0.05$) in IL-1β concentration in weeks two, three, four, five, six, eight, nine and ten in *C. pseudotuberculosis* inoculated group, whilst in MAs inoculated group, IL-1β concentration increased significantly ($p<0.05$) in weeks three, five, six, eight, nine and ten compared to the control. However, IL-1β showed significant decrease ($p<0.05$) in week twelve in both treated groups. The concentration of IL-6 was significantly increased ($p<0.05$) in weeks five, six, seven and eight in *C. pseudotuberculosis* inoculated group compared to the control, whilst MAs inoculated group indicated a significant increase ($p<0.05$) in weeks two, three, four, five, six and eight compared to the control. Therefore, this study concluded that MAs extracted from *C. pseudotuberculosis* has immunogenic properties represented by all the significant changes in concentration of IL-1 β and IL-6.

Keywords: *Corynebacterium pseudotuberculosis*, Mycolic Acid, Immune Response, Cytokines, Goats

Introduction

Caseous Lymphadenitis (CLA) is a widely spread disease all over the world in sheep and goats. It is found in Australia, Asia, Europe, Africa and Americas (Jesse *et al*., 2008; Komala *et al*., 2008). Farmers encounter severe economic downturn in meat production after an inspection which clearly indicates that affected carcasses must be condemned in abattoirs (Williamson, 2001; Praveena *et al*., 2010; Abdullah *et al*., 2015). CLA

causes weight loss, decline milk, wool and meat production, carcass condemnation and skin rejection in abattoirs (Dorella *et al*., 2006; Jesse *et al*., 2016). Many experimental study on the infection of *C. pseudotuberculosis* have been carried out intraperitoneally, intravenously, intranasal, intratracheally, intravaginally, orally and intradermally (Othman *et al*., 2014; Abdullah *et al*., 2015). However, no information available on the effect of MAs extracted from *C. pseudotuberculosis* (Jesse *et al*., 2016).

The leukocytes produce cytokines, different forms of cytokines regulate immunity, hematopoiesis and inflammation (Hoffman *et al*., 2001). They also regulate quite a number of other physiological and pathological functions (Jin *et al*., 2007). Inflammatory cytokines are divided into functional classes and they are primarily lymphocyte growth factors. Other forms function as pro-inflammatory or anti-inflammatory molecules whereas other cytokines polarize the immune response to antigen. For the past 25 years, cytokines have become a key tool in diagnostic roles in medicine and therapeutics in human disease (Moore *et al*., 2001).

Cytokine plays a role in the development of pyogranulomas, which is very important in reducing the dissemination of bacteria and they are a functional parameter that determines the output of immune responses in bacterial infection (Pepin *et al*., 1997; Martoriati *et al*., 2002). Interleukin-6 is a type of cytokines which performs quite a wide range of physiological functions including cell survival, immune and inflammatory responses (Jazayeri *et al*., 2010). Cytokine expression was very high in sheep with pyogranulomas in the draining lymph nodes as compared to those without pyogranulomas (Pepin *et al*., 1997). Chrousos (1995), Ehrhart-Bornstein *et al*. (1996), Gonzalez-Hernandez *et al*. (1994) and Reichlin (1993) stated that increased in IL-1, IL-6 and Tumor Necrosis Factor (TNF) influences the activities between the pituitary adrenal cortex axis and the host immune system.

MAs found within this bacteria exerts cytotoxic effects (Hard, 1975) and can be purified from *C. pseudotuberculosis* and it was used in an experimental study in mice (Gotoh *et al*., 1991). Gotoh *et al*. (1991) reported the effects of MAs such as inducing clinical signs in the subject. The MAs is an important virulence factor of *C. pseudotuberculosis* as they contribute to its high tenacity and its ability to persist as a facultative intracellular parasite (Baird and Fontaine, 2007). Two different biovars of *C. pseudotuberculosis* can be distinguished, based on the different nitrate reduction abilities. Strains isolated from small ruminants tend not to reduce nitrate to nitrite whereas isolates from horses and cattle almost invariably possess a nitrate-reductase and are, therefore, able to reduce nitrate (Butler *et al*., 1986; Cerdeira *et al*., 2011). The MAs is immunogenic and it might increase the proinflammatory factors. The information on MAs related with the effects on proinflammatory cytokines is still scarce. This study will provide a better understanding of the effect of MAs on the proinflammatory cytokines. The aim of this study is to investigate the effects of MAs extracted from cultures of *C. pseudotuberculosis* on proinflammatory cytokines including IL-1β and Il-6 in experimental goats.

Materials and Methods

Ethical Approval

The experimental procedure was approved to be conducted under the Animal Care and Use Ethics Committee (IACUC no: R046/2015), Universiti Putra Malaysia as required by the Animal Welfare Act (2015) in Malaysia.

Bacteria and Mycolic Acid Extraction

The *C. pseudotuberculosis* strain used in this study was originally isolated from chronic clinical case of CLA at TPU, UPM goat farm (Jesse *et al*., 2008). The isolates were then sent to the Veterinary Laboratory Service Unit, Department of Veterinary Pathology and Microbiology at the Universiti Putra Malaysia for identification and confirmation. MAs, was extracted following the method described by (Daffe and Etienne, 1999).

Experimental Inoculations Protocol

Twelve crossbred Boer goats aged between 16 and 20 months with an average weight of 30±5 kg were screened for this study. These animals were subdivided randomly into 3 different groups, with the first group containing 4 female goats inoculated with phosphate buffered solution intradermally as a control (2 mL). The second group also contain 4 female's goats were inoculated with 2 mL of MAs (1 mg mL^{-1}) intradermally, the third group consisted of 4 female goats which were inoculated with 2 mL of *C. pseudotuberculosis* 1×10^{9} cfu intradermally. Blood collections was done via jugular vein puncture at the first 24 h thereafter it continued for every 24 h post-inoculation for the first seven days of the experiment. Subsequently, the blood collection was done twice a week up to 12 weeks of post-inoculation.

Evaluation of Cytokine (IL-1β and IL-6)

Interleukin concentrations (IL-1β and IL-6) were evaluated in goat serum using ELISA kits that were commercially purchased (Cat no. QY-E140037 and QY-E140039 respectively, Qayee-Bio, Bejing, China) website: http://www.qayeebio.com. The kit was a double antibody sandwicth ELISA, a one step process assay and was conducted according to the manufacturer's instructions. The ranges were between 12.5-800 and 7.8-500 pg mL^{-1} for IL-1β and IL-6 respectively. The linear regression coefficient $r\geq0.99$. Optical Density (OD) value was calculated at 450 nm

using a plate reader. Duplicate samples were performed including control and standard. All procedures were carried out at room temperature.

Statistical Analysis

A statistical software known as JMP (version 9.0.1 SAS Institute Inc., Cary, NC, USA) was used to analyze the data obtained. The repeated measure Analysis of Variance technique was further used to analyze IL-1β and IL-6 cytokines. Values that were obtained were considered significant at $p<0.05$ and reported as a mean ± standard error.

Results

Cytokine IL-1β

Goats inoculated with *C. pseudotuberculosis* showed that concentration of cytokine IL-1β significantly increased ($p<0.05$) in weeks two, three, four, five, six, eight, nine and ten, whilst in MAs inoculated group increased significantly ($p<0.05$) in weeks three, five, six, eight, nine and ten as compared to the control (Table 1). In *C. pseudotuberculosis* treated group, the concentration of IL-1β was increased 2 folds in weeks four, five, six, eight, nine and ten, whereas in MAs treated group, IL-1β concentration showed 2 folds' increase in weeks five and ten only. However, IL-1β showed significant decrease ($p<0.05$) in week twelve in both treated groups.

Cytokine IL-6

Concentration of cytokine IL-6 was significantly increased ($p<0.05$) in weeks five, six, seven and eight in *C. pseudotuberculosis* inoculated group as compared to the control, whilst MAs inoculated group indicated a significant increase ($p<0.05$) in weeks two, three, four, five, six and eight as compared to the control (Table 2). The concentration of IL-6 peaked in week seven showing 3 folds' increase as compare to the control.

Table 1. Interleukin IL-1β response in goat's post-inoculation with *C. pseudotuberculosis* and MAs

	Cytokine IL-1β pg/mL (Mean ± SE)		
Weeks	Control –ve (A)	MAs (B)	*C. pseudotuberculosis* (C)
0	62.76±2.07^{ab}	59.68±4.33^{b}	57.07±4.811^{b}
1	77.07±3.89^{b}	90.44±17.21^{ab}	92.27±4.20^{ab}
2	65.82±7.52^{c}	96.73±20.98^{ab}	123.99±9.82^{a}
3	66.58±5.57^{b}	126.57±3.67^{a}	116.37±6.94^{a}
4	76.72±5.64^{b}	91.72±13.98^{b}	188.12±16.41^{a}
5	65.71±5.40^{b}	165.38±33.40^{a}	186.36±44.80^{a}
6	80.23±7.47^{c}	144.80±49.91^{b}	207.75±27.14^{a}
7	80.96±6.90^{b}	96.03±5.58^{ab}	98.61±21.75^{ab}
8	71.19±10.27^{c}	100.21±29.58^{b}	163.94±41.79^{a}
9	67.44±14.93^{c}	111.95±32.46^{b}	148.08±45.43^{a}
10	63.22±12.02^{c}	120.14±23.40^{b}	160.96±38.69^{a}
11	65.72±9.83^{ab}	56.19±14.47^{b}	54.21±29.58^{b}
12	67.13±10.09^{ab}	51.21±29.58^{a}	47.71±14.42^{a}

^{abc}All values were expressed a significant ($p<0.05$) comparison between inoculated groups i.e., control, Mycolic acid and *Corynebacterium pseudotuberculosis*

Table 2. Interleukin IL-6 response in goat's post-inoculation with *C. pseudotuberculosis* and MAs

	Cytokine IL-6 pg/mL (Mean ± SE)		
Weeks	Control –ve (A)	MAs acid (B)	*C. pseudotuberculosis* (C)
0	57.61±1.44^{b}	78.32±1.44^{ab}	74.73±1.85^{ab}
1	70.95±0.85^{b}	86.82±0.80^{ab}	77.20±3.58^{ab}
2	82.32±2.38^{b}	97.44±2.89^{a}	87.65±1.01^{b}
3	71.62±0.77^{b}	108.55±1.57^{a}	85.37±2.73^{ab}
4	65.27±2.06^{b}	85.27±2.06^{a}	73.43±1.36^{b}
5	55.77±1.92^{c}	84.21±2.51^{b}	111.01±6.61^{a}
6	65.32±1.49^{c}	94.48±1.57^{b}	187.67±4.76^{a}
7	73.81±1.94^{b}	87.21±2.18^{b}	231.02±1.63^{a}
8	73.72±1.03^{c}	99.76±2.31^{b}	117.44±0.67^{a}
9	84.38±1.32^{a}	80.32±1.06^{a}	86.84±3.60^{a}
10	82.62±2.37^{a}	81.37±3.45^{a}	87.69±1.70^{a}
11	81.25±3.85^{a}	81.87±0.44^{a}	80.19±0.99^{a}
12	74.31±1.68^{a}	84.96±0.69^{a}	71.34±1.69^{a}

^{abc}All values were expressed a significant ($p<0.05$) comparison between inoculated groups i.e., control, Mycolic acid and *Corynebacterium pseudotuberculosis*

Discussion

There remains a dearth of information of IL-1β and IL-6 response in goats in relation to *C. pseudotuberculosis* and MAs. This experiment therefore evaluated the response of IL-1β and IL6 to *C. pseudotuberculosis* and MAs challenges in crossbred Boer goats. Pro-inflammatory cytokines are very important mediators of inflammatory responses which is involved in cellular activities that include the proliferation of cells, differentiation and the programmed cell death (Othman *et al*., 2014). Jesse *et al*. (2016) reported that goats exposed to *C. pseudotuberculosis* showed significant increase in proinflammatory cytokines concentrations especially IL-1β and IL-6. Similarly, in this study, both IL-1β and IL-6 concentrations were significantly stimulated in both treated group. Elevated level of IL-1β indicates the acute nature of the early infection of *C. pseudotuberculosis* as well as the chronic stage. Meanwhile, IL-6 increased level one month post challenge signifies and verifies the chronic nature of CLA disease (Jesse *et al*., 2016). The delayed response of IL-1β in MAs treated group (started from week two up to week ten) may indicate the immunogenic property of MAs. In addition, IL-1β delayed response may also indicate the involvement of cellular immunity. Hence, IL-1β known as "leukocytic pyrogen" indicating that MAs has pyrogenic properties that led to elevated level of IL-1β. Moreover, IL-1β function is to activate lymphocytes which is a vital step toward cellular immune response (Marrakchi *et al*., 2014).

Dinarello (1998) stated that IL-1β neutralization resulted in reduction of systemic and localization of the disease conditions in goats. It is possible that the elevated concentration of IL-β1 in this study is related to the severity of the disease. Similarly, the increased IL-1β showed that infection by *C. pseudotuberculosis* has taken place. This finding is supported by (Jesse *et al*., 2016) who also indicated that bacterial infection stimulated the defense mechanism of the host thereby leading to an increase in the concentration of proinflammatory cytokines IL-1β. In addition, the present study is also in agreement with (Othman *et al*., 2014) who showed that the IL-1β was the earliest cytokine to respond to *C. pseudotuberculosis* inoculation at early stage.

The precise mechanism by which MAs influenced IL-6 concentration is not yet been established. However, our study showed that IL-6 was significantly increased in MAs treated group. In addition, MAs induced earlier response in IL-6 level compare to *C. pseudotuberculosis*. In contrast, the finding reported by (Kishimoto *et al*., 1995) indicated that the concentration of IL-6 elevated in infectious, inflammatory diseases and in cancer related diseases. The latter findings are in harmony with those findings reported by Othman who stated that there was a significant increase in IL-6 concentration post inoculation with *C. pseudotuberculosis* (Othman *et al*., 2014).

Commonly, cytokine IL-6 have been documented to be affected by *C. pseudotuberculosis* in laboratory animals, sheep and goats (Jesse *et al*., 2016). Nonetheless, there is still lack of data on MAs effects on this cytokine. The rise of IL-6 in the present investigation suggests that IL-6 level corresponds with the severity of the disease condition. Hence, MAs inoculated group showed significant increase in IL-6 concentration earlier. We hypothesized that MAs is involved in the innate immune response of the host and the sensitization of lymphocytes to produce antibodies against MAs. Of all, (Kaplanski *et al*., 2003) stated that IL-6 is the main cytokine that involves in the change of acute to chronic inflammation. The elevated concentration of IL-6 concentration may be due to severity of disease in does challenged with *C. pseudotuberculosis*. It is also involved in the development of specific cellular or humoral immune responses including immunoglobulin secretion and the activation of T cells. The latter reports show that MAs has immunogenic properties that play a key role in CLA pathogenesis.

Conclusion

C. pseudotuberculosis and MAs showed significant changes in the cytokine concentration for IL-1β and IL-6. In addition, this study concludes that MAs plays a key role in pathogenesis of CLA disease in small ruminants. Furthermore, we conclude that MAs has immunogenic properties that may induce immunoglobulins production.

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Author's Contributions

Faez Firdaus Jesse Abdullah: This experimental study had contributions from Jesse FFA and Mohammad Naji Odhah. It was conducted at the experimental unit of the Department of Veterinary Clinical Studies, University Putra Malaysia.

Zaid Khudher Mahmood: Sample collection and approval of manuscript.

Odhah Mohd Naji: Drafting of initial manuscript sampling and statistical analyses.

Abd Wahid Haron: Corrected and approve final manuscript.

Mohd Azmi Mohd Lila: Statistical analysis corrected and approved.

Mohd Zamri Saad: Conceived the idea for research and approved final manuscript.

Idris Umar Hambali: Sample collection and approval of manuscript.

Ethics

For "Ethics" as indicated just before the "reference" section, the "Ethical Approval" under "Materials and Methods" covers for that query.

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