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Short Communication

Immune Responses of Goats to *Corynebacterium pseudotuberculosis* and its Mycolic Acids (MAs) Extract

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Abstract | *Corynebacterium pseudotuberculosis* is the causative agent of caseous lymphadenitis in small ruminants and is of considerable economic importance in many countries worldwide. The control of the disease in animals depends on the control of the disease in infected animals. Nevertheless, few countries where the disease was previously endemic have successfully eradicated the pathogen. This investigation was undertaken to determine the antibody titre (IgM and IgG) in does challenged with *C. pseudotuberculosis* and its Mycolic acids (MAs) immunogen extract. About 12 healthy crossbred female Boer goats were assigned into three groups (A, B and C), each comprising of 4 goats. Group A (Negative control group) was inoculated intradermally with 2 ml of sterile phosphate-buffered saline (PBS-pH 7); Group B (Mycolic acid group) was inoculated intradermally with 2 ml of immunogenic Mycolic acid extract (1g /ml); while group C (Positive control group) was inoculated intradermally with 2 ml of 10^9 colony-forming unit of live *C. pseudotuberculosis*. All the animals were observed for 90 days post-inoculation. Blood samples were collected via the jugular vein from all the groups before the inoculation and once weekly after the challenge until the end of the research period. The result of the study showed that the Immunoglobulin M (IgM) concentration in goats inoculated with *C. pseudotuberculosis* significantly ($p<0.05$) increased at week 2 (18.97±0.28 ng/ml), week 3 (33.97±0.59 ng/ml), week 4 (31.94±0.28 ng/ml) and week 5 (12.51±2.42). Whereas, in the mycolic acid group, the IgG antibody titre increased consistently from week 1 to week 3 (20.99±1.96 ng/ml; 15.52±3.78 ng/ml; 14.74±0.34 ng/ml) respectively. On the other hand, the Immunoglobulin G (IgG) concentration was found to significantly increase ($p<0.05$) after treatments and the increase persisted up till week 11 post-inoculation. The concentration of IgG steadily increased in *C. pseudotuberculosis* and MAs-treated groups, reaching the peak at week 9 (32.82±8.56 ng/ml) in *C. pseudotuberculosis* group and week 10 (28.41±1.27 ng/ml) in MAs group, then declined slowly from week 10 till week 12. In conclusion, there was an elevated level in the IgM and IgG antibodies post-infection with *C. pseudotuberculosis* and MAs. However, while the IgG antibody levels started declining at week 9 for *C. pseudotuberculosis*, the MAs group was sustained until week ten before reducing. Therefore, MAs can be used as an immunogen to control caseous lymphadenitis infection caused by *C. pseudotuberculosis* among small ruminants.

Keywords | *C. pseudotuberculosis*; Mycolic acid; Caseous Lymphadenitis (CLA); Antibodies IgM and IgG; Immune responses; Goats

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INTRODUCTION

Caseous lymphadenitis (CLA) is a chronic infectious disease that has an affinity for sheep and goats (Baird & Fontaine, 2007; Abdullah et al., 2019). The causative agent *C. pseudotuberculosis* is an intracellular gram-positive bacteria (Batey, 1986; Mahmood et al., 2016). The infection (CLA) occurs in two distinct forms; the external and internal forms (Lopez et al., 1966; Jesse et al., 2017). This infection has a complex immune pattern which involves a synergy between the two types of the immune response (Ellis et al., 1991; Faeza et al., 2019b). However, the cell-mediated immune response is the predominant immune response in sheep and goats with CLA compared to the humoral antibody response (Pepin et al., 1997; Alves & Olander, 1998; Odhah et al., 2018). Several studies have been conducted to understand the immune response of sheep and goat with caseous lymphadenitis, yet the understanding with respect to the immunity is still vague. Nonetheless, the humoral immunity has been recently reported to be highly essential during CLA in sheep and goats (Baird & Fontaine, 2007; Odhah et al., 2017; Faeza et al., 2019a).

The extraction of the Mycolic acid (MAs) fraction of the bacteria can be achieved using organic solvents or via the terminal esterification of the Penta-arabinofuranosyl units of arabinogalactan (McNeil et al., 1991). Mycolic acid of *C. pseudotuberculosis* is a geometric and positional dioenoic isomer (Parodi, 1999; Abdullah et al., 2019). It is composed of alkyl-b-hydroxyl fatty acids of a specific length and complexity which represents a major lipid component of the cell wall of some bacteria including *C. pseudotuberculosis* and *M. tuberculosis* among others. The cell wall purified mycolic acid MAs has been shown to mimic certain vital features of natural infections with the actual bacteria as earlier reported (Korf et al., 2005; Odhah et al., 2019).

The intracellular mycolic acid is capable of triggering signaling pathways leading to the activation and differentiation of infected macrophage, and subsequent endocytosis of excreted mycolic acid-containing complexes may trigger the activation and differentiation of the cell, thereby promoting an immunological response (Korf et al., 2005; Odhah et al., 2018).

The immunoglobulin M (IgM) is a component of the humoral immune response elicited by T-independent (TI) antigens**.** It is known to play a significant role in microbial immunity (Robbins et al., 1995). Unlike earlier reports indicating the short half-life of IgM, it has recently been demonstrated that IgM produces prolonged response post-infection or immunization (Vos et al., 2000). It is present in high concentrations in blood and can neutralize a broad range of pathogenic bacteria or viruses due to its high acidity. While immunoglobulin G (IgG), on the other hand, is considered the most effective humoral response to many protein antigens. It has two antigen-binding sites and represents the most abundant form of immunoglobulin in circulation. The molecules are produced and released by B cells following invasion by a pathogenic microorganism (Mond et al., 1995; Abdullah et al., 2015). The IgM is by far the physically most abundant antibody, and it is the first antibody secreted following exposure to an antigen. It is also one of the most sensitive acute phase markers of inflammatory and infectious conditions in ruminants. It is routinely used to assess the innate immune response to vaccination in sheep (Selim et al., 2010; Othman et al., 2014b; Odhah et al., 2018).

IgM and IgG are among the most important parameters that serve as markers during the early and later stage of CLA. Observations of the kinetics of these two acute/chronic phase markers in the development and progression of CLA lesion is necessary for immunological response post-infection with *Corynebacterium pyogenes* (Eckersall et al., 2008; Dorella et al., 2009; Saad et al., 2013). Nonetheless, IgM as marker gives an earlier detection of the infection than the IgG (Maki et al., 1985) and it possesses a key role in CLA (Perkins et al., 2004). Many studies have, however, been conducted within the study area, but there remains a gap in information on the immune response to *C. pseudotuberculosis* and its mycolic acid. This study, therefore, seeks to investigate the humoral immune response after experimental challenge with *C. pseudotuberculosis* and MAs.

MATERIALS AND METHODS

Ethical statement

This study was conducted in accordance with the approval of the Institutional Animal Care and Use Committee (IACUC), Universiti Putra Malaysia (UPM/IACUC/AUP-R46/2015).

Bacterial inoculum

A stock of *C. pseudotuberculosis* isolated from transudate of skin lesion from infected goats was obtained from the Taman Pertanian Universiti (TPU) repository, Universiti Putra Malaysia. The bacterial stock was cultured on a 10% sheep blood-MacConkey's agar and incubated at 37°C for 48 hours. Bacterial colonies were morphologically identified (as small, white, dry, and crumbly colonies) and biochemically confirmed by the fermentation of sugars (glucose, xylose, maltose, and sucrose), catalase reaction, urease reaction and nitrate reduction tests (Othman et al., 2016). The identified *C. pseudotuberculosis* colonies were suspended in normal saline and used to prepare a concentration of 10^7 CFU/ml using the McFarland technique (Mcfarland, 1907).

Mycolic acid inoculum

The mycolic acid of *C. pseudotuberculosis* was extracted according to the method of Brogden and Engen (1990) with a slight modification. Briefly, colonies of *C. pseudotuberculosis* were inoculated into 250 ml of brain heart infusion broth containing 1% Tween 80 and then incubated at 37 °C in a shaking incubator for 24 hours. Subsequently, the broth culture was then centrifuged at 6000 rpm at 4°C for 15 minutes to sediment the bacterial cells. The supernatant was decanted, and the bacterial pellets were washed twice with sterile distilled water, followed by 50% acetone, 100% acetone, and then finally washed twice in 100% diethyl ether before leaving to air-dry. The mycolic acid was then extracted according to the method described in previous studies (Daffe and Etienne, 1999).

Experimental design

The sample size for this study was calculated based on assumptions of 80% power in a three-group, two-sided *t*-test with a Type I error rate of 0.05. A total of twelve (n=12) healthy non-pregnant adult female Boer crossbred goats were used for the study. The goats were initially acclimatized for two weeks in the animal experimental house, Faculty of Veterinary Medicine, Universiti Putra Malaysia. During acclimatization, the animals were treated for bacterial infections (by a single dose of 20% oxytetracycline at 1ml/10kg body weight) and parasitic infections (by a single subcutaneous injection of 1% Ivermectin). The goats were given a feed ration comprising of Napier grass, commercial goat pellet and water *ad libitum*. The goats were assigned to 3 groups of 4 each designated as; Group A which represent the negative control group was given 2 ml intradermal injection with sterile phosphate-buffered saline (PBS pH 7); Group B represents the mycolic acid group and was also given 2 ml intradermal injection containing 1g /ml of the immunogen Mycolic acid extract; while Group C treated by an intradermal injection of 2 ml of 10^9 colony-forming unit (CFU) of *C. pseudotuberculosis*. All the animals were observed for a period of ninety days after the inoculation. Blood samples were collected from the animals from the jugular vein at the beginning of the experiment before the inoculation, and then subsequently twice weekly after inoculation until the 12th week.

Determination of serum IgG and IgM by ELISA

The QAYEE-BIO® goat immunoglobulin M (IgM) (Cat. No. QY-E140049) and IgG (Cat No. QY-E140013) double-antibody sandwich ELISA test kits were used for the quantitative determination of serum antibodies against *Corynebacterium pseudotuberculosis* and its mycolic acid extract in experimental animals. The assay procedures were performed under controlled conditions according to the manufacturer's instructions (QAYEE-BIO® Shanghai, China). The optical density (OD) of blank, standard, and unknown wells of each plate were measured at 450nm wavelength using a microplate ELISA reader (Tecan® Sunrise, MA, USA). The concentration of IgM and IgG was calculated using a four-parameter logistic curve fit (www.myassays.com).

Statistical Analysis

Experimental data was summarized in Microsoft® Excel Spread Sheet program version 2016 and analyzed with GraphPad prism version 8.0. The effects of different treatments and time periods on serum IgM and IgG were analyzed by two-way analysis of variance. Dunnett's two-sided test was used to compare the mean serum antibodies between different treatments and the control at 5% level of significance.

RESULTS

Immunoglobulin M (IgM)

The mean values of IgM concentration of all treated groups are shown in Table 1. The concentration of IgM was found to be significantly ($p<0.05$) increased at week 2 (18.97±0.28 ng/ml), week 3 (33.97±0.59 ng/ml), week 4 (31.94±0.28 ng/ml) week 5 (12.51±2.42) post inoculated with *C. pseudotuberculosis* compared with the control, and a significant increased ($p<0.05$) in week 1 (20.99±1.96 ng/ml), week 2 (15.52±3.78 ng/ml), week 3 (14.74±0.34 ng/ml) post-inoculation with MAs compared to the control (5.01±1.64 ng/ml).-

Table 1: Means (±S.E) of IgM concentration (ug/ml) of the inoculated group throughout the study period

Experimental groups			
Weeks	**Negative control**	**Mycolic acid**	***C. pseudotuberculosis***
0	7.63±0.94	6.72±1.33	7.18±1.81
1	7.49±0.84^{c}	20.99±1.96^{a}	13.70±0.51^{b}
2	6.44±0.73^{c}	15.52±3.78^{b}	18.97±0.28^{a}
3	7.50±0.40^{c}	14.74±0.34^{b}	33.97±0.59^{a}
4	6.57±0.95^{b}	7.75±2.40^{b}	31.94±0.28^{a}
5	6.15±0.80^{b}	6.21±0.84^{b}	12.51±2.42^{a}
6	7.02±1.17^{b}	7.67±0.75^{b}	9.94±1.38^{ab}
7	7.97±2.05	8.20±1.09	8.16±0.42
8	5.65±0.53^{b}	6.00±0.92^{b}	6.80±1.41^{ab}
9	6.90±0.99^{b}	6.50±0.78^{b}	7.80±0.80^{ab}
10	6.31±0.93	5.59±0.95	6.34±1.40
11	5.01±1.64^{c}	5.61±1.17^{b}	6.39±0.86^{ab}
12	5.43±0.78^{c}	7.77±0.70^{ab}	5.47±0.75^{c}

Means with different superscripts (^{a,b,c}) within rows indicate a statistical significance at $p<0.05$.

Table 2: Means (S.E) of IgG concentration (ug/ml) of the inoculated group throughout the study period

Experimental groups			
Weeks	**Negative control**	**Mycolic acid**	***C. pseudotuberculosis***
0	7.54±0.18	7.51±0.73	8.37±0.42
1	8.16±0.85^{c}	14.72±2.37^{ab}	12.16±2.05^{b}
2	8.16±0.66^{c}	17.20±2.85^{b}	19.93±4.57^{a}
3	10.99±0.57^{c}	20.91±3.20^{b}	26.69±9.50^{a}
4	8.54±0.94^{c}	22.70±2.82^{a}	19.66±2.59^{b}
5	11.99±1.54^{c}	21.13±1.95^{ab}	20.29±5.12^{ab}
6	14.06±1.09^{c}	25.84±6.13^{ab}	27.43±4.71^{a}
7	11.79±1.20^{c}	21.31±6.32^{a}	19.51±8.47^{ab}
8	9.04±1.54^{c}	19.09±4.44^{b}	29.76±5.19^{a}
9	11.67±1.93^{c}	28.41±1.27^{b}	32.82±8.56^{a}
10	12.14±2.26^{c}	26.94±2.93^{b}	31.17±9.03^{a}
11	6.64±0.34^{c}	17.39±5.59^{b}	19.82±3.70^{ab}
12	7.41±0.21^{c}	17.32±5.07^{b}	18.96±4.30^{ab}

Means with different superscripts (^{a,b,c}) within rows indicate a statistical significance at $p<0.05$.

Immunoglobulin G (IgG)

Table 2 is a representation of IgG mean concentration of the experimental groups. The concentration of IgG in *C. pseudotuberculosis* and MAs groups significantly increased ($p<0.05$) after all treatments and persisted till week 12 post-inoculation. The concentration of IgG steadily increases in *C. pseudotuberculosis* and MAs treated groups reaching peak concentration at week 9 (32.82±8.56 ng/ml) in *C. pseudotuberculosis* group and (28.41±1.27 ng/ml) in MAs group, then declines slowly till week 12.

DISCUSSION

The causative agent of caseous lymphadenitis is *C. pseudotuberculosis*. The disease is a source of considerable economic hardship among small ruminant farmers. Despite the economic and public health importance of this disease, information on its pathogenesis and an immune response is still lacking in addition to unavailability of a satisfactory specific treatment regime (Dorella et al., 2009; Jesse et al., 2013b). This present study evaluated the involvement and magnitude of the humoral immune responses following challenge with *C. pseudotuberculosis* and MAs in female goats. This study provided primary data, which is an at-

tempt to study the stimulatory effects of *C. pseudotuberculosis* and MAs induction on the humoral immune response as well as the determination of its impact on the antibodies in infected goats.

The result of the current study indicates that IgM titre in goats challenged with *C. pseudotuberculosis* and MAs group was considerable showing significant responses. Significant changes with respect to the antibody titre were observed on day 7 post-MAs inoculation, but the highest level was recorded on day 15 and beyond post challenged. This is in contrast with other studies where high concentration of IgM was observed at the early phase of the experiment. Nonetheless, the disparity observed from the result of this study may be attributed to the virulence of the bacteria used as demonstrated by the humoral response induced by the antigen specific IgM antibodies which renders protection against the intracellular bacterial infections including *Mycobacterium tuberculosis* and *Nocardia brasiliensis* (González et al., 2008). Worthy if note is the fact that the organisms are both intracellular pathogens with similar features to *C. pseudotuberculosis*, which happens to be another member of the same family. Although MAs can stimulate the secretion of IgM, their activity is not reflective of the specific immune response following tuberculosis infection as typified by the low affinity of the IgM and the cross-reactivity in addition to its pentameric structure.

The *Corynebacterium pyogenes* antigen induced a robust immunological response observed from day 7 to day 21 post-immunization. The result of our study also indicated that goats with high IgM titres are likely to have less risk of developing CLA abscesses compared to animals with low IgM levels. Several studies on IgM antibody levels following *C. pseudotuberculosis* infection has been reported. However, our observation agrees with earlier studies that demonstrate the protective effect of the humoral antigen-specific IgM response in preventing the progression of CLA in sheep (Bastos et al., 2013).

These results are in conformity with studies that experimentally infected small ruminants, indicating that a transient IgM level was seen a few days post-infection with *C. pseudotuberculosis* (Bastos et al., 2013). The MAs also showed significant increase in IgM in week 1, 2 and 3. This study positioned that MAs alone has less capability to stimulate IgM production as like *C. pseudotuberculosis*. Furthermore, the significant effect of *C. pseudotuberculosis* on IgM may be due to the bacterium itself and its virulent factors, including MAs and PLD.

Goats in *C. pseudotuberculosis* group had a significant mark in the rise of IgM when compared with goats in MAs group, which had an elevated level. Objectively, this is an indication that activated whole-cell *C. pseudotuberculosis* antigen was immunogenic. The present finding is in harmony with previous studies where CLA infection was characterized by an early immune (IgM) response (Bastos et al., 2012).

Antibody-mediated immunity is the main body defence against extracellular pyogenic (pus-forming) organisms. Commonly, antibodies are secreted after the stimulation of B lymphocyte. The antigen-binding receptor on a B lymphocyte is an immunoglobulin (IgG). Following the stimulation of the B lymphocytes, the B cell proliferates and differentiates in to plasma cell which the releases specific memory B cells that permits the immune system to respond rapidly in event a similar antigen is encountered in the future (secondary response).

Generally, IgG antibody was more sensitive according to Julián et al. (2002) after their comparative studies of IgG, IgM, and IgA antibody responses based on four trehalose-containing glycolipids, obtained from patients infected with *Mycobacterium tuberculosis* (Julián et al., 2002).

The IgG levels of goats in *C. pseudotuberculosis* and MAs inoculated groups was significantly increased in the early phase. The peak of IgG titre was seen day 14 post-inoculation in both *C. pseudotuberculosis* and MAs groups which then gradually declines toward the end of the study. On the other hand, antibody response to *C. pseudotuberculosis* and MAs treated groups was high when compared to the control.

Therefore, the results of the current experimental study extrapolate that there is a great possibility of rapid response to *C. pseudotuberculosis* whole-cell antigen in the early phase of the experiment. Our findings have been concurring with previous studies that assessed humoral response to CLA disease and attenuated strain T1 (Moura-Costa et al., 2008). This study also reported that the increased in the titres of IgG at the early stage of *C. pseudotuberculosis* and MAs inoculation. Moreover, it is also in harmony with the reports of Paule et al. (2003) who estimated the kinetics of IgG which were observed between days 11 and 21 post-inoculation with *C. pseudotuberculosis* (Paule et al., 2003). These results agree with Eckersall et al. (2007) reported a significantly high level of IgG post-inoculation with *C. pseudotuberculosis* in sheep.

Furthermore, the adjuvanted protein fraction of *C. pseudotuberculosis* has been demonstrated to show significant IgG based humoral immune response. The experiment also reported that there was a significant increase in the humoral immune response after challenged. This is similar in the present study, where it demonstrated an increasing

level of IgG production from day 14 to day 90 after immunization with a significant level of protection.

Other studies indicated that antigen *C. parvum* at 1×10^9 was immunogenic (Ghaffar & Sigel, 1978). However, antigen dose had been sought to not be an issue because as low as 56 mg of *C. parvum* significantly evoke both IgM and IgG response. The present findings also correlate with those mentioned above and can serve a base for the prevention of CLA. MAs demonstrates the potential to attract and activate the immune system during challenge following real exposure thereby leading to an elevated response (Goren, 1982; Bhatt et al., 2007).

The results of *C. pseudotuberculosis* and MAs treated groups where IgM and IgG levels showed double fold, and there is, therefore, every possibility that the challenge had a prolonged positive effect on the innate immune response during the acute phase period.

CONCLUSION

Conclusively, the effect of *C. pseudotuberculosis* and MAs induced a considerable humoral immune response. Therefore, this study has shown that CLA resistance is proportional to the increased of IgM and IgG in the early acute phase responses of post challenged with *C. pseudotuberculosis* and MAs. Additional knowledge and a new understanding of immune responses towards *C. pseudotuberculosis* and its immunogen mycolic acid in goats were elucidated.

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CONFLICT OF INTEREST

There is no conflict of interest.

NOVELTY STATEMENT

This study elucidates the role of mycolic acids of C. pseudotuberculoisis as a potential vaccine candidate for the control of caseous lymphadenitis among sheep and goat flocks.

AUTHOR'S CONTRIBUTIONS

The experimental study was conducted at the experimental unit of the Department of Veterinary Clinical Studies, University Putra Malaysia. FFJA, contributed to the conceived and planned the experiment. MNO and FNMN provided guidance during some aspect of laboratory experiments and result analysis, BG and ZMK contributed to sample preparation, KA, AM and WH contributed to the interpretation of the results. MAML and MZS assisted in the development and editing of the manuscript. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

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