

Acute Phase Response in Mice Experimentally Infected with Whole Cell and Exotoxin (PLD) Extracted from *Corynebacterium pseudotuberculosis*

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Abstract: Acute Phase Proteins (APP) are blood proteins that contribute to restoring homeostasis and limiting microbial growth in an antibody-independent manner in animals subjected to infection, inflammation, surgical trauma or stress. There are still lack of knowledge of acute phase protein profiles in mice associated with infection of *Corynebacterium pseudotuberculosis* and its exotoxin Phospholipase D (PLD). In this study, serum concentrations of three different positive Acute Phase Proteins (APPs) are studied; Serum Amyloid A (SAA), Haptoglobin (Hp) and α_1 Acid Glycoprotein (AGP). This study was conducted to acquire a better way of understanding the pathophysiology response of *Corynebacterium pseudotuberculosis* and its exotoxin in mice model. A total of 48 mice, 2-3 weeks of old both sexes were enrolled and equally divided into three groups; namely control, whole cell and exotoxin groups. Mice of whole cell groups were exposed intraperitoneally to 1 mL of the inoculums containing 10^9 Colony-Forming Unit (CFU) mL of live *C. pseudotuberculosis*. Exotoxin group were infected intraperitoneally with a single dose of exotoxin (PLD) extracted from *C. pseudotuberculosis*. While control group were exposed intraperitoneally to 1 mL of Phosphate-Buffered Saline (PBS), pH 7. Blood samples were collected by cardiac puncture for acute phase protein analysis. All APPs were quantified by commercially available ELISA methods and AGP was assessed by highly sensitive clourometric assay. The results revealed that there were statistically significant differences ($p < 0.05$) between APPs concentrations throughout the experimental period in groups of mice induced with whole cell and exotoxin of *C. pseudotuberculosis* compared to control groups. The concentrations of Hp and SAA were significantly induced after infection of *C. pseudotuberculosis* with mean maximum levels from days 1-4 of post infection whereas the AGP concentration was significantly induced after 3rd day of post infection. About >70 fold increase was observed in Hp concentrations after experimentally induced whole cell and single intraperitoneal dose of exotoxin whereas SAA and AGP increased <15 fold. No significant differences ($p > 0.05$) were observed in acute phase proteins profiles between whole cell and exotoxin groups. Therefore, the results of this study indicated that acute phase proteins can be used as potential biomarkers for assessing caseous lymphadenitis.

Key words: *C. pseudotuberculosis*, exotoxin (PLD), SAA, α_1 -AGP and Hp, Malaysia

INTRODUCTION

Corynebacterium pseudotuberculosis is a causative agent of chronic infections in a number of different mammalian species, the most significant of which is Caseous Lymphadenitis (CLA) or cheesy gland, a chronic granulomatous infectious disease of sheep and goats that is characterized by the formation of abscesses, typically located in superficial lymph nodes and lungs. Phospholipase D (PLD) has been identified as a potent exotoxin in *C. pseudotuberculosis* and a key virulence factor in the development of CLA.

Acute Phase Proteins (APP) are a group of non-structurally related proteins present in blood which modify their concentrations in attempt to help contribute restoring homeostasis and limiting microbial growth in an antibody-independent manner in animals subjected to infection, inflammation, surgical trauma or stress (Murata *et al.*, 2004). The physiological response of APP to these conditions and the initiation of events leading to a systemic response is known as Acute Phase Reaction (APR). Measurement of APPs is widely used in human medicine (Berbari *et al.*, 2010; Patel *et al.*, 2010; Sage *et al.*, 2010) and there is growing interest in their use

in companion animals (Eckersall, 2010). Recently, monitoring the circulating serum levels of these proteins has become of clinical importance in determining the presence and also the extent of tissue damage caused by an infectious disease as their levels are related to the severity of the underlying condition. Research suggests that in the future, APPs measurements may routinely be used in farm animal production to assess animal health, optimize production rates or monitor antibiotic therapy (Eurell *et al.*, 1992; Lipperheide *et al.*, 2000; Dickhofer, 2002; Lauritzen *et al.*, 2003; Eckersall and Bell, 2010). Serum Amyloid A (SAA), Haptoglobin (Hp) and α_1 Acid Glycoprotein (AGP) have been relatively established as a major acute phase proteins in both companion and farm animals. However, there have to date been limited studies carried out using experimental infections of *C. pseudotuberculosis* that are mainly involved to some extent in visceral tissue damage of animals subjected to this devastating microorganism. There is therefore a need to characterize the Acute Phase Response (APR) in mice infected with whole cell and exotoxin extracted from *C. pseudotuberculosis*. This would facilitate the evaluation of potential use of acute phase proteins in the assessment of *C. pseudotuberculosis* infections and in monitoring response to possible future clinical therapy. In addition to that, this would shed light on the possible role of acute phase proteins in the pathogenesis of *C. pseudotuberculosis* infections. As mice have been used in preference to sheep for their susceptibility and economic feasibility (Cameron *et al.*, 1969). These laboratory models, therefore have been used to evaluate the response of these biomarkers following infection of *C. pseudotuberculosis* immunogens prior to the application to large herbivores in the field.

MATERIALS AND METHODS

Animals: All procedures and experiments described were undertaken under a project license approved by Animal Utilization Protocol Committee with reference number: UPM/FPV/PS/3.2.1.551/AUP-R120. Briefly, these studies had used 48 apparently healthy mice, 2-3 weeks of old. The mice were randomly divided into three groups namely control, whole cell and extoxin group where the first group of mice were intraperitoneally inoculated with 1.0 mL of sterile Phosphate Buffer Solution (PBS), pH 7, the second group of mice were intraperitoneally inoculated with 1.0 mL of 10^6 colony forming unit (cfu) of live *C. pseudotuberculosis* and the third group of mice were intraperitoneally inoculated with 1.0 mL of single dose of exotoxin (PLD) extracted from *C. pseudotuberculosis*.

Animals were maintained under the same condition where they were kept in the stocking density of 5 mice/cage in an air conditioned room, fed with commercial mice pellets and drinking water which were freely available for an acclimatization period of 1 week before the beginning of the study.

Inoculum preparation (*C. pseudotuberculosis*): Blood agar culture made from a lymph node that was naturally infected with caseous lymphadenitis was earlier culturally and biochemically identified as *C. pseudotuberculosis*. These isolated were revived by subculturing onto newly prepared blood agar 24 h at 37°C. The cultures were then harvested and suspended in normal saline solution where concentration estimated to the standard dose of 1×10^6 CFU mL⁻¹ using the Mac Farland technique.

Innoculum preparation of Toxin (PLD): About 250 mL of frozen toxin which was earlier extracted from the organism (*C. pseudotuberculosis*) were thawed prior inoculation.

Collection of blood samples: Just prior the sacrificing using cervical dislocation, blood samples were collected for hematology and biochemistry profiling, by cardiac puncture using a 26G×1.5 Venoject needle (PrecisionGlide™, Becton Dickinson, UK) with venoject holder (Vacutainer®, BD vacutainer™, USA).

Assays of acute phase proteins

Serum Amyloid A (SAA): Serum SAA concentrations ($\mu\text{g mL}^{-1}$) were determined using commercial ELISA kits (Tridelta PHASE™ RANGE Mouse SAA Elisa kit, Tridelta Development, Ltd. Wicklow, Ireland). The assay was performed according to the manufacturer's instructions. Serum samples were initially diluted 1:500. Samples obtained from challenged groups and the standards were tested in duplicate. Control samples were measured in triplicate and a blank (assay buffer only) measured in quadruplicate. Samples reading outside the range of the standard curve were diluted further and reassayed. The absorbance was measured at 450 nm using the FLUOstar OPTIMA platereader (BMG Labtech Ltd. Aylesbury, UK). The mean absorbance for each sample, control or calibrator was then calculated. The absorbance of the calibrators was plotted against the SAA concentration on Cartesian graph paper. Furthermore, the best smooth curve through these points was drawn to construct the calibration curve. Finally, the concentrations of the test samples and controls were determined from the calibration curve by multiplying the interpolated value by the appropriate dilution factor (1:500).

Haptoglobin (Hp): Plasma Hp concentration (mg/mL) was measured using a colorimetric assay (PHASE™ Haptoglobin Assay Cat. No. TP-801, Tridelta Development, Ltd. Wicklow, Ireland) and performed according to the manufacturer's instructions with all steps carried out at room temperature. Samples were tested neat and all samples including the standards were run in duplicate. Samples with an optical density outside the range of the standard curve were diluted further and reanalyzed. Optical densities were read on an automatic plate reader (Dynatech Laboratories, GB) at 450 nm. The mean absorbance for each sample, control or calibrator was then calculated. The absorbance of the calibrators was plotted against the HP concentration on Cartesian graph paper. Additionally, the best smooth curve through these points was drawn to construct the calibration curve. Actual concentrations of the test samples and controls were determined from the calibration curve.

Mouse alpha 1 acid glycoprotein: A commercial kit (Mouse Alpha 1 Acid Glycoprotein Assay (AGP) Cat. No. TP 805M, Tridelta Development, Ltd. Wicklow, Ireland) was used for plasma α -1 AGP measurement. The assay was performed in duplicate according to the manufacturer's protocol with all steps carried out at room temperature.

Statistical analysis: The Statistical Package SPSS Software 17 (PASW Version 17; Inc., Chicago, IL, USA) was used. APP values were summarized and subjected to Analysis of Variance (ANOVA). Treatment and time were included as fixed factors. An error level of 0.05 was used.

RESULTS

Acute phase protein findings

Concentrations of Serum Amaloid A (SAA): Mean values of SAA concentrations obtained from mice infected with whole and exotoxins of *C. pseudotuberculosis* are outlined in Table 1. The infected groups were compared with control group. Statistically significant differences ($p < 0.05$) between the three different groups were observed. SAA showed considerably higher concentrations ($p < 0.05$) in infected group compared to control group. There was no difference in the concentrations of SAA depending on the character of inoculum in the mice. Total mean of SAA concentrations in control whole challenged and toxin (PLD) of *C. pseudotuberculosis* were 19.06 $\mu\text{g mL}^{-1}$ (SD = 10.83), 197.50 $\mu\text{g mL}^{-1}$ (SD = 44.83) and 169.68 $\mu\text{g mL}^{-1}$ (SD = 52.16), respectively.

Table 1: SAA concentrations ($\mu\text{g mL}^{-1}$) of challenged and non challenged mice (Mean \pm SD)

Time (h)	Treatment		
	Control	<i>C. pseudotuberculosis</i>	Toxin (PLD)
12	20.00 \pm 7.07 ^{a,x}	160.00 \pm 7.07 ^{b,x}	185.00 \pm 14.14 ^{b,x}
24	12.50 \pm 3.53 ^{a,x}	225.00 \pm 7.07 ^{b,x}	235.00 \pm 7.07 ^{b,x}
48	35.00 \pm 14.14 ^{a,x}	235.00 \pm 14.14 ^{b,x}	195.00 \pm 21.21 ^{b,x}
72	22.50 \pm 10.60 ^{a,x}	260.00 \pm 14.14 ^{b,x}	207.50 \pm 17.67 ^{b,x}
96	17.50 \pm 3.53 ^{a,x}	200.00 \pm 14.14 ^{b,x}	80.00 \pm 21.21 ^{b,x}
120	7.50 \pm 3.53 ^{a,x}	215.00 \pm 21.21 ^{b,x}	155.00 \pm 56.56 ^{b,x}
144	10.00 \pm 7.07 ^{a,x}	145.00 \pm 35.35 ^{b,x}	152.50 \pm 53.03 ^{b,x}
168	27.50 \pm 10.60 ^{a,x}	140.00 \pm 7.07 ^{b,x}	147.50 \pm 45.96 ^{b,x}
Total	19.06 \pm 10.83	197.50 \pm 44.83	169.68 \pm 52.16

Table 2: Haptoglobin concentration (mg mL⁻¹) of challenged and non challenged mice (Mean \pm SD)

Time (h)	Treatment		
	Control	<i>C. pseudotuberculosis</i>	Toxin (PLD)
12	0.05 \pm 0.07 ^{a,x}	0.90 \pm 0.07 ^{b,x}	1.90 \pm 0.70 ^{c,x}
24	0.00 \pm 0.00 ^{a,x}	1.50 \pm 0.28 ^{b,x}	2.20 \pm 0.70 ^{c,x}
48	0.07 \pm 0.10 ^{a,x}	2.25 \pm 0.35 ^{b,x}	2.15 \pm 0.21 ^{b,x}
72	0.01 \pm 0.00 ^{a,x}	2.70 \pm 0.28 ^{b,x}	1.55 \pm 0.28 ^{c,x}
96	0.02 \pm 0.00 ^{a,x}	2.25 \pm 0.14 ^{b,x}	2.57 \pm 0.10 ^{b,x}
120	0.00 \pm 0.00 ^{a,x}	1.45 \pm 0.35 ^{b,x}	0.82 \pm 0.17 ^{c,x}
144	0.00 \pm 0.00 ^{a,x}	1.42 \pm 0.39 ^{b,x}	1.17 \pm 0.03 ^{b,x}
168	0.02 \pm 0.00 ^{a,x}	1.45 \pm 0.42 ^{b,x}	2.50 \pm 0.28 ^{c,x}
Total	0.02 \pm 0.04 ^{a,x}	1.74 \pm 0.61 ^{b,x}	1.85 \pm 0.67 ^{b,x}

Table 3: AGP concentration (mg mL⁻¹) of challenged and non challenged mice (Mean \pm SD)

Time (h)	Treatment		
	Control	<i>C. pseudotuberculosis</i>	Toxin (PLD)
12	267.00 \pm 3.53	637.50 \pm 3.5300	345.00 \pm 35.350
24	262.00 \pm 3.53	950.00 \pm 70.710	355.00 \pm 7.0700
48	272.00 \pm 3.53	900.00 \pm 141.42	505.00 \pm 7.0700
72	255.00 \pm 7.07	900.00 \pm 141.42	770.00 \pm 28.280
96	267.00 \pm 3.53	950.00 \pm 70.710	975.00 \pm 37.350
120	265.00 \pm 7.07	900.00 \pm 42.420	495.00 \pm 7.0700
144	265.00 \pm 7.07	985.00 \pm 21.210	275.00 \pm 35.350
168	270.00 \pm 0.00	710.00 \pm 14.140	265.00 \pm 14.140
Total	265.62 \pm 6.29	866.00 \pm 133.92	498.12 \pm 245.46

^{a-c}Means with different superscripts with in row differed significantly ($p < 0.05$) due to treatment effect. ^{x-z}Means with different superscripts with in column differed significantly ($p < 0.05$) due to time effect

Concentrations of Haptoglobin (HP): Mean values of haptoglobin concentration in the different groups studied are shown in Table 2. There was highly significant difference ($p < 0.05$) in the mean concentrations of haptoglobin between the infected and non infected groups two. Haptoglobin mean values in the total of animals studied were 0.02 mg mL⁻¹ (SD = 0.04), 1.74 mg mL⁻¹ (SD = 0.61) and 1.85 mg mL⁻¹ (SD = 0.67) respectively.

Concentrations of Alpha 1 Acid Glycoprotein (AGP): Data of AGP concentrations for all mice tested were summarized in Table 3. There was significant difference in AGP concentrations between challenged and non-challenged mice. Within infected groups, AGP levels were

shown to be significantly higher in animals infected with *C. pseudotuberculosis* than in mice infected with exotoxin (PLD). Among the whole organism group, the highest level of AGP was obtained at 144 h post-infection where levels of this protein increased almost 4 fold.

DISCUSSION

To the researchers knowledge this is the first study to comprehensively evaluate the APR of mice to infection with *C. pseudotuberculosis*. The current study of experimental nature was undertaken to analyze whether APP serum concentration (Hp, SAA and AGP) were associated to the severity of infection induced by whole cell and exotoxin (PLD) of *C. pseudotuberculosis* and be potentially useful as quantitative biomarkers of infection, inflammation and potential diagnostic tools in clinical settings. A relevant point of the present study was that a small sample size of mice was analyzed, independently of their individual categories and significant differences were found between mice classified according to three types of groups. Overall, no research has been carried out about the relationship between serum APPs and infection of either *C. pseudotuberculosis* or the exotoxin extracted. Therefore, the current findings reported for the first time that Hp, SAA and AGP can be used unspecific markers and potential reference of experimental infections of live and exotoxin extracted from *C. pseudotuberculosis*. Raised levels of AGP, SAA and Hp were observed in this study where AGP and SAA considered useful biomarkers since their concentrations had a very high statistical significance in experimentally animals. These findings were constant with results obtained from feline acute phase response (Selting *et al.*, 2000; Correa *et al.*, 2001). It is very important to note that the mice involved in the present study, more specifically challenged ones, showed clinical signs of infection ranged between mild to severe. Mice of no apparent clinical signs, control group had lower concentrations for Hp, SAA and AGP. The concentration of Hp, SAA and AGP were significantly increased in animals challenged both *C. pseudotuberculosis* and exotoxin (PLD). However, very little is known about the acute phase protein kinetics response against caseous lymphadenitis. Nevertheless, in an experimental infection model mimicking infection by interpretoneal inoculation with *C. pseudotuberculosis* in mice, Hp, SAA and AGP responded with large increases in serum concentrations at the acute phase of this disease although, the increase, kinetics of induction and normalization were different between these proteins in many studies. Thus, the three APPs involved in this study did show together a prolonged response after the

challenge. This latter finding may be related to the higher concentrations of Hp, SAA and AGP described in the current research which dealt with the chronic consequences of this disease (CLA) in theoretically clinically healthy mice.

CONCLUSION

In this study, serum concentration of APPs are affected by several factors which included age, breed, housing, management conditions, disease and prolonged transportation (Saco *et al.*, 2003; Pineiro *et al.*, 2007, 2009). All mice included in the current findings were approximately of the same age and strain. Data about housing, management conditions and transport distance to the UPM laboratory of clinical studies were carefully collected as reported by Fraile *et al.* (2010). Therefore, it is assumed that differences in serum concentrations of APPs in the present study were mainly a consequence of the pathological conditions affecting the animals.

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