

Clinico-pathological response of mice following oral route infection of *C. pseudotuberculosis*

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Abstract: *Corynebacterium pseudotuberculosis* is a facultative, rod shaped, gram-positive bacteria which is the causative agent of caseous lymphadenitis (CLA). CLA is a chronic granulomatous infectious disease that is characterized by formation of abscesses typically located in superficial lymph nodes and lungs. Difficulties in early diagnosis of CLA infected animals have limited the effectiveness of controlling and eradicating the disease. The aim of the present study was to acquire a better way of understanding the pathogenesis of CLA via oral route infection in a mouse model. A total of sixteen healthy mice (n=16) were divided equally into 2 groups, where the first group of mice were orally inoculated with 1.0 mL of sterile phosphate buffer solution (PBS), pH 7, and the second group of mice were orally inoculated with 1.0 mL of 10⁹ colony forming unit (cfu) of *C. pseudotuberculosis*. Clinical signs and histopathological changes in visceral organs were compared between the diseased and non-diseased group within the period of 120 hours of post inoculation. Results of this study showed that there were no significant (P>0.05) changes in clinical signs observed between diseased and non-diseased group. However, there were significant (P<0.05) histopathological changes observed in the visceral organs (lung, liver and kidney) between diseased and non-diseased group where the visceral organs had severe congestion and increased vascularization, micro-abscesses formation, infiltration of neutrophils and macrophages, tubercular granulomas, necrosis and early signs of degeneration as compared to the non-diseased group.

Keywords: *C. pseudotuberculosis*, caseous lymphadenitis (CLA), mice, clinical response, cellular changes

I. Introduction

Caseous lymphadenitis (CLA) is common and chronic disease that prevalent in the entire world's major sheep and goat production areas [1-3]. It is mainly characterized by the formation of abscesses, typically located in superficial lymph nodes (cutaneous form), and internal lymph nodes and organs (visceral form) [4-6]. The causal pathogen of CLA is *Corynebacterium pseudotuberculosis*, a rod shape, gram-positive, non-spore forming bacteria. It is a facultative intracellular parasite that is found on fomites, in soil and manure contaminated with prudent exudate [7]. Because of the chronic, subclinical nature and the presence of the viable bacteria inside capsulated abscess of infected animals, the control of the disease (CLA) with antimicrobial or antibiotics becomes completely challenging [8, 9, 1]. Thus, its prevalence is rising particularly in developing countries. However, the disease causes significant economic losses in the small ruminant industry [10]. In Malaysia, CLA is a potential threat to the livestock industry causing several negative impacts on both productivity and fertility of small ruminant herds.

Therefore, this study was conducted to acquire a better way of understanding CLA in subclinical cases through mouse model. Furthermore, there is a lack of information on the pathogenesis and clinical signs of CLA in mouse model via oral route infection. Moreover, information on host cellular response in mice following oral route inoculation of *C. pseudotuberculosis* is insufficient. Thus, the objective of the present study was to determine whether there were similar developments of clinical signs together with pathological changes in mice following the oral route inoculation with reference to actual cases that occur in small ruminants (sheep and goat).

II. Materials And Methods

Animals

Sixteen Balb/c mice of both sexes, aged 3–4 weeks were obtained from Institute of Cancer Research (ICR), Kuala Lumpur, Malaysia. The mice were housed at the Animal Experimentation Unit (AEU), which was

maintained at 21–23 °C. As they were kept in stocking density of 4 mice/cage, fed a commercial lab animal pellet and provided water which was freely available for an acclimatization period of 1 week before the beginning of the studies. All experiments were conducted under protocols approved by the Animal Experimentation Ethics Committee (AEEC), Faculty of Veterinary Medicine, Universiti Putra Malaysia (UPM).

Bacteria

A stock culture of *C. pseudotuberculosis* made from a lymph node that was naturally infected with caseous lymphadenitis from previous outbreak of CLA in the animal farm of University Putra Malaysia (UPM) was used to prepare the inoculums. The Organism was cultured onto blood agar and incubated at 37°C for 24h. Thereafter, the organism was sub-cultured in Brain Heart Infusion (BHI) broth for 24 h and concentration were estimated to the standard dose of 1×10^9 colony-forming units/ml (CFU) using the Mac Farland technique.

Experimental design

The experiment was carried out in two separate groups; A (non-diseased; n=8) and B (diseased; n=8), making the total 16 mice in the experiment. Each mouse in the non-diseased group (A), were orally inoculated with 1.0 mL of sterile phosphate buffer saline (PBS), pH 7. In contrast, each mouse in the diseased group (B), were orally inoculated with 1.0 ml of 10^9 colony forming unit (CFU) of *C. pseudotuberculosis*. Within 120 hours after inoculation, clinical signs and mortality rates were observed in both groups. Mice showed severe clinical signs and survived mice after 120 hours post infection was sacrificed via cervical dislocation and post mortem examination was performed. Tissue samples were obtained from heart, lungs, lymph node, kidney, intestine and brain for histopathological study.

Clinical signs and Scoring

The clinical observations presented by the diseased and non-diseased groups were regularly documented from the start of the study until the end point of the experimental period. Scoring system was used to score the observational clinical signs. In summary, the clinical signs of the two groups (A & B) were scored in scale of 0-3 based on the presence of following parameters: ruffled hair, eye discharge, movement and responsiveness. The score 0 represented no abnormality of clinical signs observed, 1 for mild, 2 for moderate, 3 for severe. The evaluation method of the scoring system was summarized in Table I.

**Table I
Clinical Observation Scoring System**

Parameters	Clinical Score			
	0	1	2	3
Ruffled hair	Normal fur	Ruffled fur by 30% of the body	Ruffled fur by 60% of the body	Ruffled fur more than 60% of the body
Eye discharge	No discharge from the eyes	Eye discharge by 30%	Eye discharge by 60%	Eye discharge more than 60%
Movement	Normal movement and appetite	Reduced movement by 30%	Reduce movement by 60%	reduce movement more than 60%
Responsiveness	Normal responsiveness	Reduced responsiveness by 30%	reduce responsiveness by 60%	reduce responsiveness more than 60%

Histopathology and lesion scoring

Mice showed severe clinical signs and survived mice after 120 h post infection was sacrificed via cervical dislocation and post mortem examination was performed. Tissue samples were collected from the heart, lung, lymph nodes, liver, kidney, intestine and brain. Tissues were fixed with 10% formalin except for the brain tissue samples which were fixed with 40% formalin and processed in an automatic tissue processor and was routinely stained by haematoxylin and eosin for histopathology evaluation. Microscopically, cellular changes were scored following observation of 5 slides per organ. Six microscopic fields for each slide were observed at 200x magnification. Lesion scoring was divided into 4 scores; namely: score 0: normal (normal), score 1: mild (less than 30% of field involved), score 2: moderate (30-60% of field involved) and score 3: severe (more than 60% of field involved).

Statistical analysis

The findings of the study were analysed by using the independent T-test. Significant difference between mean value of diseased group and non-diseased group was analysed using the SPSS version 19.

III. RESULTS AND DISCUSSION

Clinical Signs

Animals both in diseased and non-diseased groups exhibited no clinical signs. Thus, the differences in the clinical scores of both groups were not significant ($P>0.05$) in the following parameters; ruffled hair coats, eye discharge, movement/depression and responsiveness.

These findings were not in agreement with those reported by [11]. In their findings, significant clinical signs in certain parameters such as ruffled hair, for instance, was found in mice that were inoculated intraperitoneally with *C. pseudotuberculosis*. Similarly, our findings differed from the experimental study conducted by [12], in which they stated that in some cases, infections with *C. pseudotuberculosis* do produce clinical signs. However, this could be attributed to the short period of observation (5 days) of the study and the effectiveness of different inoculation routes to produce significant clinical symptoms. Longer experimental period may normally allow diseases course (CLA) to be developed effectively and progression to be observed in other organs [13].

Histopathological findings

Microscopical examination of the visceral organs (Liver, lung & Kidney) of the infected group revealed significant histopathological changes. The observed lesions were microscopically scored and summarized in Table II. Infiltration of neutrophils, macrophages, congestion, increased vascularisation, and haemorrhages in the alveolar lumen bronchiole and formation of micro abscesses were observed microscopically in lungs of the infected group (Fig. 1 & 2). Similar lesions were observed in the liver of mice treated with *C. pseudotuberculosis* where Infiltration of neutrophils and macrophages, degeneration, vacuolation (necrosis), haemorrhage and formation of micro abscesses were observed (Fig. 3 & 4). In mice treated with *C. pseudotuberculosis*, kidney showed evidence of haemorrhages, degeneration, congestion and tubular necrosis (Fig. 5-8).

The histopathological lesions observed in this study were similar to other experimental studies associated with CLA infections through different inoculation routes in sheep and goats. The findings of the present study were also consistent to those reported by [11]. However, the route of the infection differed in both studies. The hispathological lesions in the lung, liver and kidney do vary in response to the route of infection and character of inoculum; phospholipase D (PLD) and mycolic acid [14]. Phospholipase D (PLD), an exotoxin produced by *C. pseudotuberculosis*, is a potent exotoxin that promotes the hydrolysis of ester bonds in mammalian cell membrane, resulting in the damage or destruction of host cell membranes, which may further lead to their dysfunction or disruption [15]. Moreover, PLD causes dermonecrotic lesions and is lethal to different species of laboratory and domestic animals at high doses [16]. Unlike PLD, mycolic acid also plays a major role in causing histopathological lesions in visceral organs. The production of localized swelling with congestion and necrosis often in the central area of haemorrhagic necrosis was reported in experimental infections with mycolic acid extracted from *C. pseudotuberculosis* [17]. Other experimental studies, using sheep and goats as models infected with *C. pseudotuberculosis*, showed the presence of pyogranulomatous lesions [18]. The less frequently infected tissues include heart, brain, spinal cord, testes, uterus and joints [18]. The spread of infection in the host might occur by the transfer of the bacterium via the blood or lymphatic system, resulting in the formation of lesions at other loci within the host [1]. Our results were in agreement with the experimental study conducted by [13] where infections with *C. pseudotuberculosis* developed lesions in liver, lungs, and kidney. In contrast, the extent of the severe lesions observed by [13] and [11] were not present in the present work. In this study, similar cellular changes were observed but the difference in the extent of the severity of the cellular changes may due to the short period of this study.

Table II
Scoring system for the lesions of visceral organs of mice after 120 h post inoculation with *C. pseudotuberculosis*.

Organs	Groups	Parameters				
		Oedema	Inflammatory cells	Degeneration/ Necrosis	Hemorrhage	Kupffer Cell
Lung	Control	0.08±0.02	0.10±0.04	0.08±0.02	0.12±0.06	NA
	Treated	1.38±0.97*	1.58±0.93*	1.46±0.72*	1.25±0.85*	N/A
Liver	Control	-	0.08±0.02	0.08±0.02	0.12±0.06	0.06±0.01
	Treated	-	1.58±0.65*	1.29±0.46*	1.21±0.66*	1.33±0.56*
Kidney	Control	-	0.06±0.01	0.08±0.02	0.12±0.06	N/A
	Treated	-	1.25±0.68*	1.17±0.64*	1.50±0.51*	N/A

*Significant value $p<0.05$. N/A=not applicable

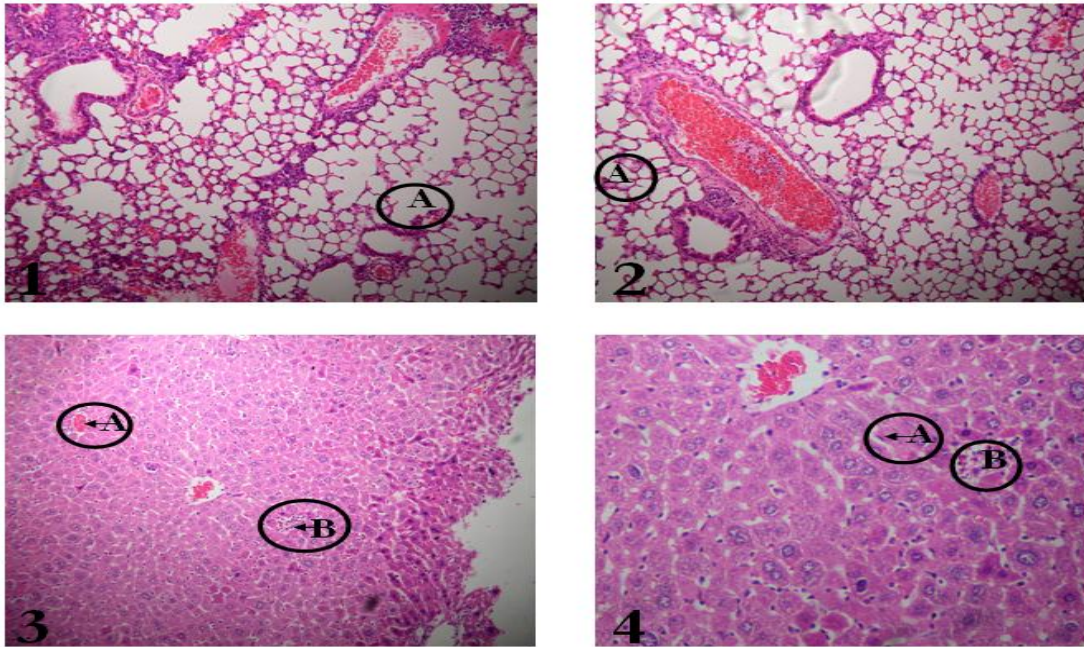


FIGURE 1.—Photomicrograph of a section of lung of mouse inoculated with *C. pseudotuberculosis*.Part of vascularization of the lung (A) (HE, X200). FIGURE 2.—Photomicrograph of a section of lung of mouse inoculated with *C. pseudotuberculosis*. Congested blood vessel lumen and massive influx of inflammatory cells (A) (HE, X200). FIGURE 3.—Photomicrograph of a section of liver of mouse inoculated with *C. pseudotuberculosis*. Markedly congested (A) and inflammatory cells (B) (HE, x100). FIGURE 4.—Photomicrograph of a section of liver of mice inoculated with *C. pseudotuberculosis*. Kupffer Cell (A) and Micro-abscesses formation (B). (HE, x200).

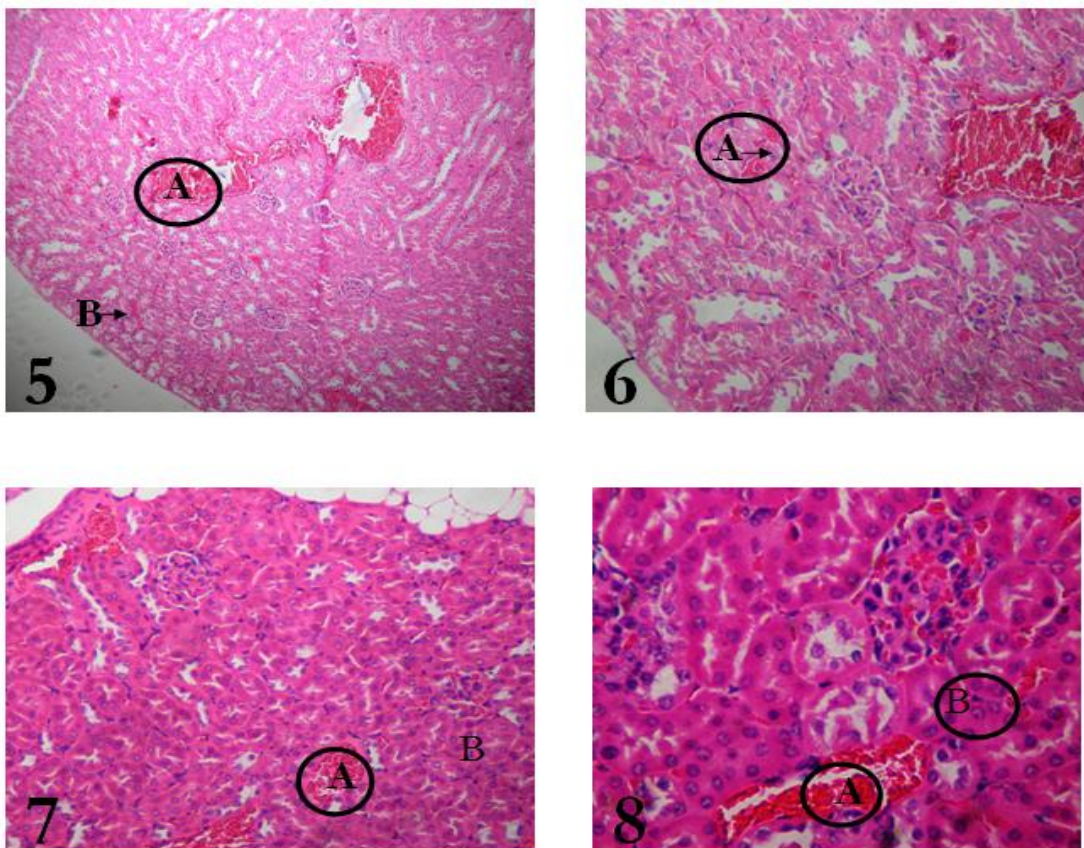


FIGURE 5.—Photomicrograph of a section of kidney of mouse inoculated with *C. pseudotuberculosis*. Congestion (A).Intertubular haemorrhage (B) (He, x100). FIGURE 6.—Photomicrograph of a section of kidney of mouse inoculated with *C. pseudotuberculosis*. Severe intertubular haemorrhage and early signs of degeneration (A) (He, x200). FIGURE 7.—Photomicrograph of a section of kidney of mouse inoculated with *C. pseudotuberculosis*.Severe congestion (A). Signs of early degeneration (B) (He, x 200). FIGURE 8.—Photomicrograph of a section of kidney of mouse inoculated with *C. pseudotuberculosis*. Severe congestion (A). Signs of early degeneration (B) (He, x 400).

IV. Conclusion

This study revealed that oral exposure of live *C. pseudotuberculosis* resulted relatively establishment of CLA infection in a mouse model. The oral inoculation of *C. Pseudotuberculosis* in mice failed to produce the typical clinical signs of CLA but did produce significant histo-pathological changes that were characteristic of CLA.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect the research, authorship, and/or publication of this article.

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