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Polymerase Chain Reaction Detection of *C. pseudotuberculosis* in the Brain of Mice Following Oral Inoculation

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Abstract: The aim of present study was to detect the presence of *C. pseudotuberculosis* in the brain of the mice following oral inoculation as a model using PCR. Caseous lymphadenitis is a chronic and subclinical disease of sheep and goats which has universal distributions, presenting enormous animal and flock prevalence. Total of 16 mice were used for this study, 8 mice were inoculated orally with 1.0 mL sterile phosphate buffered saline pH 7, while another 8 mice were inoculated with 1.0 mL of 10^9 colony forming unit of *C. pseudotuberculosis*. Seven different organs were collected during post mortem for the detection of *C. pseudotuberculosis*. The result indicated 3 positive samples in lymph nodes, 5 in the brain and 1 in the liver. The PCR used in the present study may successfully be applied for the detection and diagnosis of *C. pseudotuberculosis* in the brain of the mice following oral inoculation.

Keywords: Brain, *C. pseudotuberculosis*, detection, mice, oral inoculation, PCR

INTRODUCTION

Caseous Lymphadenitis (CLA) is a chronic and subclinical disease of sheep and goats of widespread distributions, presenting huge animal and flock prevalence (Alessandro de Sá *et al*., 2011; Jesse *et al*., 2011). CLA is caused by the aetiological agent *Corynebacterium pseudotuberculosis* (*C. pseudotuberculosis*) which is an intracellular, non spore forming Gram-positive, facultative anaerobe small curved rod bacterium, its capacity for surviving for a protracted periods in the environment and its poor compliance to therapeutics are the key attributes thus, contributing to the soaring transmission rate of CLA within a herd (Williamson, 2001; Baird and Fontane, 2007; Seyffert *et al*., 2010; Jesse *et al*., 2011; Pavan *et al*., 2012). CLA is a chronic supportive necrotizing inflammation and abscesses of superficial and internal lymph nodes of both sheep and goats (Sarah *et al*., 2007; Marcilia *et al*., 2011). The bacterium is also the aetiological agent of ulcerative lymphangitis in cattle and horses and external and internal abscesses in horses (Spier and Whitcomb, 2007). Infection due to *C. pseudotuberculosis* has also been indicated in buffalos, camelids, equids and a zoonosis in humans (Selim, 2001; Anderson *et al*., 2004; Braga *et al*., 2006; Join-Lambert *et al*., 2006; Guimarães *et al*., 2009). It is commonly referred to as "Cheesy-gland" and CLA is prevalent in all the major sheep and goats rearing areas of the world (Glenn, 2000; Robert, 2004). In chronic cases, these abscesses are also seen in the internal organs, particularly the lungs, liver, kidneys and spleen, typifying visceral CLA (Carmen *et al*., 2006; Dorella *et al*., 2006; Marcilia *et al*., 2011).

The transmissions are through abscess rupture releasing huge numbers of the bacteria onto the skin and fleece, resulting in the contamination of the environment. Other animals may then be exposed to the bacteria, either through direct physical contact with the affected individual or indirectly via contaminated fomites (Fontaine and Baird, 2008).

The most important and consistent way of controlling this disease is by vaccination, detection and removal of infected animals (O'Reilly *et al*., 2010; Peter, 2011). The diagnosis of CLA is largely dependent on the features of the clinical symptoms and on the separation of the aetiological agent from discharging abscesses. Detection of the cultured organisms as *C. pseudotuberculosis* is more often than not accomplished by biochemical tests but, is frequently challenging due to extensive unpredictability in biochemical characteristics of the pathogen (Cetinkaya *et al*., 2002; Magdy *et al*., 2010). Therefore, the present study aims to detect the presence of the pathogenic *C. pseudotuberculosis* in the brain of the mice as a model following oral inoculation using Polymerase

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Chain Reaction (PCR) on Deoxyribonucleic Acid (DNA) extraction with a pair of *C. pseudotuberculosis* specific primer.

MATERIALS AND METHODS

Preparation of the bacteria: The present study was conducted in Universiti Putra Malaysia, Faculty of veterinary Medicine during the 2011/2012 Academic session of the University. The bacteria colony of *C. pseudotuberculosis* in the present study was obtained from the nutrient agar storage, which was isolated from previous outbreak of CLA in Taman Pertanian Universiti (TPU), Universiti Putra Malaysia (UPM). Identification of the bacteria was made by using gram staining and also biochemical test. Then the colony was subculture on the blood agar media and incubated at 37°C for 48 h to grow. To confirm the bacteria growth as *C. pseudotuberculosis*, the identification was done by microscopic examination which revealed Gram positive small curved rods. The colony was then cultured back in the new nutrient agar for storage. The bacteria colony in the nutrient agar was than cultured in four different blood agar to grow and for it to be use in the preparation of 10^9 colony forming unit (cfu) of *C. pseudotuberculosis* and was determined by McFarland technique for inoculation.

Mice and inoculation: Sixteen mice were divided into two equal groups consisting of 8 mice each. The mice in group 1 were inoculated orally with 1.0 mL sterile Phosphate Buffered Saline (PBS) pH 7, while group 2 was inoculated with 1.0 mL of 10^9 colony forming unit (cfu) of *C. pseudotuberculosis*. Mortality of the mice was observed over 120 h (5 days). Surviving mice after 120 h were sacrificed by cervical dislocation. All procedures and experiments illustrated were undertaken under a project license approved by Animal Utilization Protocol Committee with reference number: UPM/FPV/PS/3.2.1.551/AUP-R120.

Sampling and culture: Organ sampled were the lymph node, liver, heart, lung, brain, stomach and small intestine during the post mortem. All the samples were cultured on the blood agar media and incubated for 48 h at 37°C. After the incubation period, similar morphological characteristic which are small, white, dry and crumbly colonies were cultured back into the new blood agar for identification using PCR.

DNA extraction: DNA extraction in the present study was performed using boiling method. A few colonies from the cultures were transferred into an Eppendorf tube containing 50 uL distilled water and the suspension was boiled at 100°C for 15 min. After boiling, the suspension was immediately cooled on ice for 2 min. Then, the suspension was centrifuged at 13,000 rpm for 5 min. The upper phase was carefully transferred into another Eppendorf tube to be use as DNA template.

PCR condition: The PCR was performed in a touchdown thermocycler in a total reaction volume 10 uL of PCR buffer, $MgCl_2$, 250 uM of deoxynucleotide triphosphate, 2 U of Taq DNA polymerase and 1 uM of each forward and reverse primer and 5 uL of template DNA. Amplification was performed with 30 cycles following an initial denaturating step at 94°C for 5 min. Each cycle involved denaturation at 94°C for 1 min, annealing at 56°C for 1 min, extension at 72°C for 2 min and final extension at 72°C for 5 min.

Primer design: The primer for the amplification of the *C. pseudotuberculosis* was referenced to Cetinkaya *et al*. (2002). The forward primer use was (5'CCGCACTTTAGTGTGTGTG'3) and the reversed primer had the sequence of (5'TCTCTACGCCGATCTTGTAT'3). This set of primer will target *C. pseudotuberculosis* species specific regions 16S rRNA and the length of PCR product is 816 bp.

Agarose gel preparation: (1.5%) agarose gel was prepared; 1.5 g agarose gel powder was poured into 100 mL bijour bottle, then top up with 1% TAE to 100 mL. The mixture was heated in microwave oven for about 3-5 min until all the precipitate melted, liquid form gel was then leave to cool down to 60°C. After that it was poured into suitable size cast. Wait for the gel to solidify for about 30 min. After the gel was solidified and turns to cloudy white color the gel was then ready for PCR loading and electrophoresis.

Electrophoresis: Agarose gel was placed into the gel holder tank and submerged with 1% TAE buffer and making sure that the holding wells were near the negative terminal and the band will run towards the positive terminal. Make sure the positive and negative terminals were place properly. One hundred bp marker (Promega) was used. Five uL of PCR product was loaded into the well carefully without breaking it with the pipette. One uL of loading dye was mixed with 2 uL ladders by using pipette and loaded into the first well. The PCR were run in 1.5% agarose gel for 45 min at 81V. Than the gel was stained with ethidium bromide 0.5 ug/mL solutions and stirred for 20 min, then the gel was dip into distilled water. Lastly, the gel was placed under UV gel imaging capturing machine and the results was recorded. All the 8 mice (treatment group) in the present study survived throughout the after 120 h post inoculation. Post mortem was conducted and the following organs were sampled namely the lymph node, brain, heart, lungs, stomach, intestine and liver. All the organs were cultured on the blood agar and incubated at

Table 1: Showed the overall results for PCR detection of *C. pseudotuberculosis* in all organs of mice

No	Organ	Detection of the bacteria
1	Lymph node	Positive in mice 4, 5, 7
2	Brain	Positive in mice 3, 4, 5, 6, 7
3	Stomach	Negative in all mice
4	Intestine	Negative in all mice
5	Heart	Negative in all mice
6	Lung	Negative in all mice
7	Liver	Positive in mice 1

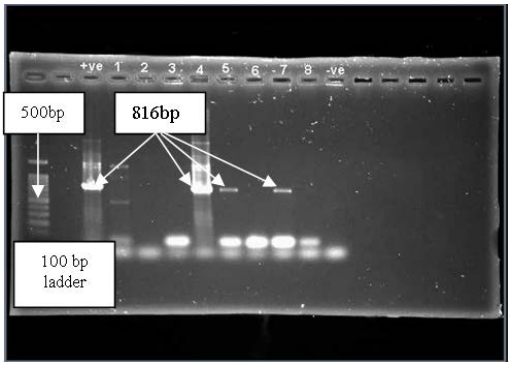

Fig. 1: PCR detection of *C. pseudotuberculosis* in lymph node

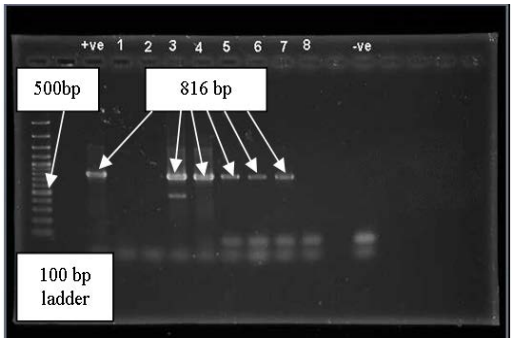

Fig. 2: PCR detection of *C. pseudotuberculosis* in brain

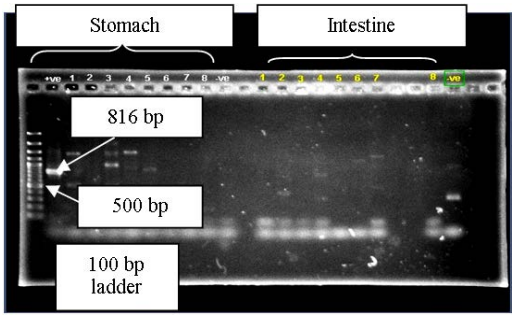

Fig. 3: PCR detection of *C. pseudotuberculosis* in stomach and intestine

37°C for 48 h. Then all the colonies with the same morphological characteristic with *C. pseudotuberculosis* were chosen for the PCR. PCR was performed on the chosen samples according to the organs from mice number 1 to mice number 8. The

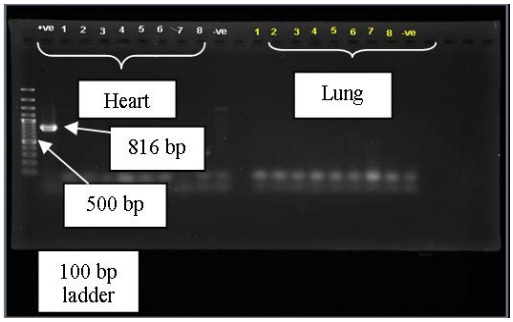

Fig. 4: PCR detection of *C. pseudotuberculosis* in heart and lung

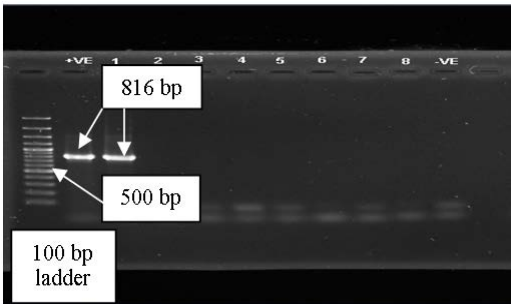

Fig. 5: PCR detection *of C. pseudotuberculosis* in liver

principle for the detection of the *C. pseudotuberculosis* is when the positive sample's lane forms the same bp size with the positive control which is 816 bp.

RESULTS

Table 1 showed the results of PCR detection of *C. pseudotuberculosis* in all the organs of the mice. Figure 1 to 5 showed the detection of *C. pseudotuberculosis* in the various selected organs using PCR.

DISCUSSION

From the results of the present study, lymph node showed positive result perhaps because lymph node is the primary replication target site for the *C. pseudotuberculosis* in CLA and this is supported by the study of Cetinkaya *et al*. (2002) which indicated similar findings in PCR detection of the bacteria from the lymph node in sheep and goats. The negative result may indicate immunity in those mice or it might be due to poor sampling of the lymph node because the samples were obtained in different locations.

To the knowledge of the authors, there is no study done to determine whether the bacteria can be detected using PCR in brain samples. In the present study, there was more positive result obtained from the brain than the lymph node which is the primary target organ. From the study conducted by Fontaine and Baird (2008), they indicated that brain is one of the least frequently affected organs in the internal form of CLA. There is

also no much information on the study done on brain lesion in CLA. In the present study, brain is one of the target organs which showed positive result than other target organs and it might be due to the pathogenesis of CLA where the bacteria start to multiply at the local or regional lymph node and spread to other lymph nodes and organs through the lymphatic or vasculature (Glenn, 2000). In the present study, oral inoculation is the route of the source of infection and the most affected lymph nodes are basically located around the head and the neck (Fontaine and Baird, 2008; Ashfaq and Campbell, 1979) such as the superficial cervical lymph nodes. From the regional lymph nodes the bacteria multiply and spread to most adjacent organs and the brain through lymphatic or vasculature system.

The heart in this study showed negative result this is because heart is one of the least frequently affected organs in the internal form of the CLA (Fontaine and Baird, 2008). The negative results may also be influenced by the protracted period of infection. Similarly, the lung is also one of the less commonly affected organs in the internal form of CLA. The lungs were usually infected through the aerosol transmission (Fontaine and Baird, 2008). Thus, the present study showed negative findings which indicate absence of *C. pseudotuberculosis* in the lung and it could be due to the oral route of inoculation and the protracted nature of the infection.

In the present study, organs such as the stomach and intestine were chosen to evaluate for the presence of the bacteria in the organs, this is because the mice were inoculated orally and the organs are the primary digestive system. In the present study, the results were negative for *C. pseudotuberculosis* detected using PCR and there were no previous study done on detection using PCR methods on these organs. Although, there was one occasion in which successful isolation of the bacteria from the stomach contents and tissues of ovine fetuses were obtained in an abortion case (Baird and Fontane, 2007). This perhaps indicates the possible existence of the bacteria in these organs.

Liver is one of the primary target organs in the internal form of CLA (Valdivia *et al*., 2012). In the present study, mice number 1 was positive in respect of the liver but, the other organs of mice number 1 were negative. This may be due to poor sampling of the other organs and also it could be due to an individual factor such as the immunity status of the animal. The other mice showed negative result and perhaps this happens as a result of the prolonged period required for the onset of infection in these organs. Thus, the present study indicated that oral route of inoculation of *C. pseudotuberculosis* will cause the same effect as other natural route of transmission. Detection of *C. pseudotuberculosis* using PCR can be efficient to facilitate the diagnosis of the organism in the brain based on the specificity and sensitivity compare to other diagnostic procedure. The present study also revealed that mice as an animal model for the study of CLA is reliable as the actual host (goat and sheep).

CONCLUSION

The oral route of *C. pseudotuberculosis* inoculation revealed successful manifestation of infection in the brain. The use of mice as a model in the study of CLA is more parsimonious than the actual host (goat and sheep). Detection of *C. pseudotuberculosis* by PCR can be efficient to facilitate the diagnosis of *C. pseudotuberculosis* in the brain based on the specificity and sensitivity.

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