

HISTOPATHOLOGICAL CHANGES IN EXPERIMENTALLY INFECTED MICE WITH PCR IDENTIFIED *Corynebacterium pseudotuberculosis*

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المستخلص

أجريت هذه الدراسة لمعرفة التغيرات النسيجية المرضية خلال المراحل المبكرة من العدوى التجريبية ببكتريا وتدية السل الكاذب في الفئران البيضاء. تم عزل بكتريا وتدية السل الكاذب من العقد اللمفية لضأن مصاب بمرض التهاب العقد اللمفية التجبني وقد أجريت هذه التجربة باستخدام خمسة عترات مختلفة في نشاطها الكيموحيوي وأكد تشخيصها بواسطة تفاعل البلمرة المتسلسل. تم إجراء التجربة باستخدام خمس مجموعات من الفئران البيضاء بكل مجموعة خمسة فئران حيث تم حقنها تحت الجلد ومراقبتها لمدة 10 أيام عقب الحقن. الفحص المرضي النسيجي أظهر أن التغيرات المرضية في الأنسجة مختلفة وتتمثل في إرتشاح بؤري لخلايا بيضاء متعددة الأنوية في الكبد والرئة والطحال وهذا يدل على وجود التهاب حاد أما في الكلى فقد وجد أن هناك ورم حبيبي متجبن ومنتخر وإرتشاح خلايا التهابية وحيدة النواة وهذا يوضح بداية تكون الآفات الحبيبية في العدوى البكتيرية المزمنة.

Abstract

This study was done to investigate the histopathological responses of the early phase during experimental *Corynebacterium pseudotuberculosis* infection in white mice. *Corynebacterium pseudotuberculosis* strains were isolated from natural cases of caseous lymphadenitis (CLA) in sheep. The experimental infection was done by using five isolates. These isolates were confirmed by PCR and they showed different biochemical characters. The experiment comprised five groups of five mice each; inoculated subcutaneously with *C. pseudotuberculosis* strains and observed for ten days following inoculation. There were different pathological involvement of the

isolates, these included; focal infiltration of polymorphonuclear cells was seen in liver, lung, spleen, which indicated an acute infection process. Typical pyogranulomas with central necrosis and peripheral mononuclear cells were observed in kidney clearly. These results illustrate the dual role of granulomatous lesions in chronic bacterial infections.

Introduction

Corynebacterium pseudotuberculosis is the causative agent of caseous lymphadenitis (CL); a disease characterized by the formation of suppurative abscesses particularly in superficial and internal lymph nodes and in internal organs in small ruminants. The disease occurs worldwide and causes significant economic losses particularly in sheep industry due to poor wool growth, decreased milk production, reproductive disorders, premature culling, carcass condemnation and rarely death (Alonso *et al*, 1992; Paton *et al*, 1994). The disease is considered to be one of the most economically important diseases of sheep and goats in Sudan, and emerged as a hurdle to the sheep export industry (Suleiman 2006). The objective of this study was to describe some aspects of pathogenicity and histopathology of PCR identified *Corynebacterium pseudotuberculosis*, during early phase of experimental infection in white mice.

Materials and Methods

Collection and Preparation of Samples:

Three thousand and seventy five sheep carcasses were examined for the presence of abscesses in lymph nodes in Al Huda slaughterhouse at Omdurman Province Khartoum State. One hundred and fifty seven grossly enlarged lymph nodes were collected from one hundred and twenty nine infected sheep carcasses. The samples were labeled, stored on ice box and transported to the Bacteriology Department at Veterinary Research Institute, Khartoum, Sudan.

Culturing and Biochemical Identification:

Pus samples from lymph node were inoculated on Blood Agar Base (Oxoid CM 271) supplemented with 7% defibrinated sheep blood. After the incubation of plates aerobically for 48 h at 37°C, small, white, dry and crumbly colonies were picked up for further identification. Pure cultures were prepared and identified according to Cowan and Steel (1974).

Identification of *Corynebacterium pseudotuberculosis* Isolates by PCR:

From forty nine isolates, five isolates were used for PCR. These were selected according to the differences in their biochemical characteristics. These isolates were 2, 11, 13, 64, and 65.

Ten milliliter of fresh culture in Brain Heart Infusion broth (Biochemick 53286) were centrifuged at 4°C (2000xg for 20min) and chromosomal DNA extraction was performed by Quiagen kits. The primer used in this study was targeted on the 16S rRNA gene of *Corynebacterium pseudotuberculosis* and the sequence was selected from previously published work (Cetinkaya *et al.* 2002) and synthesized at Vivantis Biotech. Com. (Malaysia). The length of the primers was 20 mer and the annealing temperature was 55°C.

The PCR was performed in a thermocycler (PeQlab biotechnologie GmbH, Germany) in a final reaction volume of 25µl which contained 2.5 µl of PCR Buffer, MgCl 50mM (1 µl), 1 µl of each deoxynucleotide triphosphate, 0.4µl of Tag DNA polymerase, 1µl of each primer, 2.5µl of template DNA and 15.6µl distilled water. Amplification was obtained with 30 cycles following initial denaturing step at 94°C for 5 min. Each cycle involved denaturation at 94°C for 1 min., annealing at 56°C for 1 min., and synthesis at 72°C for 2 min. The amplified products were visualized by ethidium bromide staining after electrophoresis for 1h in 1.5% agarose gel.

Experimental Infection in White Mice:

This experiment was done using the PCR –confirmed five isolates. One colony of each strain from Blood Agar plates was inoculated in 5ml of sterile Brain Heart Infusion plus 0.1% Tween 80 and incubated for 48 h at 37°C.

A dose of 0.2 ml (3.6×10^7 CFU) of fresh culture of each isolates were injected subcutaneously into five groups each of five white mice. Mice were obtained from Laboratory Animal Unit at Veterinary Research Institute.

Control group was injected subcutaneously with 0.2 ml of sterile normal saline.

Mice were observed for 10 days post inoculation for abscess development and death. Dead as well as surviving animals were necropsied and their internal organs (lung, heart, liver, spleen, and kidney) were inspected, smeared, Gram stained, and cultured on Blood Agar plates. Small parts of these organs were fixed in 10% formalin, dehydrated in a series of alcohol concentrations, embedded in paraffin wax, sectioned at thickness of 5 µm and stained with haematoxylin and eosin (H&E).

Results

On microscopic examination *Corynebacterium pseudotuberculosis* was Gram-positive and small curved rod-shaped. Isolates which were positive for catalase, glucose, O.F., maltose and urease, inhibiting beta haemolysin of *S. aureus* and negative for oxidase, lactose, V.P., and nitrate were diagnosed as *C. pseudotuberculosis*.

From one hundred and fifty seven abscesses examined in this study, *Corynebacterium pseudotuberculosis* was isolated from 49 samples (31.2%).

Identification of *Corynebacterium pseudotuberculosis* by PCR:

PCR products with the molecular size of 815 bp (Fig.1) were diagnosed as *Corynebacterium pseudotuberculosis*. The five strains were positive by PCR.

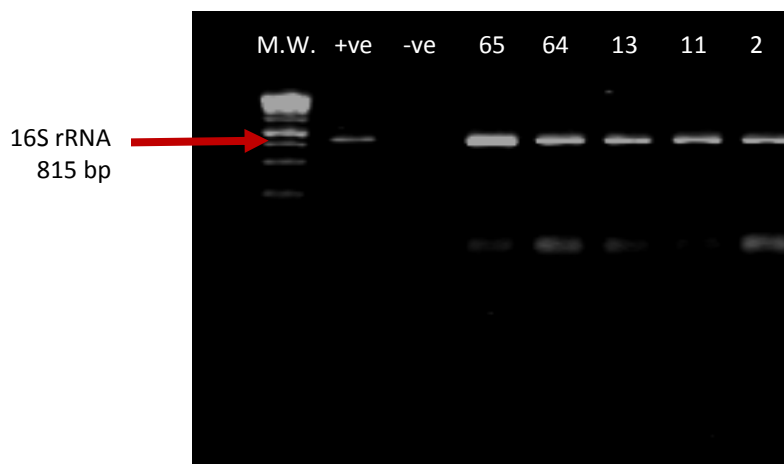


Fig.1. Identification of *Corynebacterium pseudotuberculosis* isolates by PCR

Pathogenicity:

Strains 2 and 13 were the most pathogenic as they caused acute disease and death in 2-5 days with 100% mortality rate. The organisms were demonstrated in Gram-stained smears and re-isolated from the livers, spleens, kidneys and lungs. Strains 11 and 65 were less pathogenic, caused death in 4-7 days, had mortality rates of 80% and 60% respectively and the organisms were isolated from the internal organs on Blood agar plates. Strain 64 was not pathogenic but it was isolated from liver, lung, spleen and kidney.

Post Mortem Findings:

The internal organs in all white mice were congested and the spleen was enlarged and congested.

Histopathological Findings:

Histopathological changes in all mice groups were the same, but the differences were in the severity of changes. In the kidney there was congestion, haemorrhage, granuloma and renal tubules necrosis (Fig.2). The liver showed haemorrhage, congestion, focal inflammatory cells and necrotic hepatic cells with pyknotic nuclei and cytoplasmic vacuoles (Fig.3). The spleen showed depletion of lymphocytes, giant cells and RBCs(Fig.4). In the lung, congestion, haemorrhage, thickening of alveolar septa and inflammatory cells were observed (Fig.5).

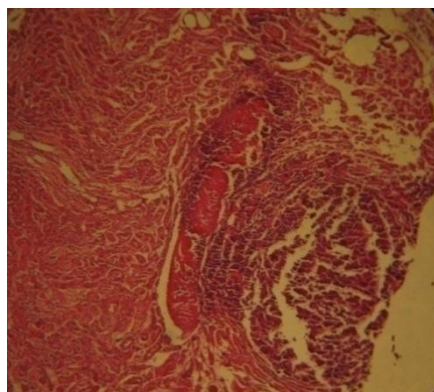


Fig.2. Congestion, heamorrhage & Granuloma in kidney of white mice infected with *C. pseudotuberculosis* H&E(X10)

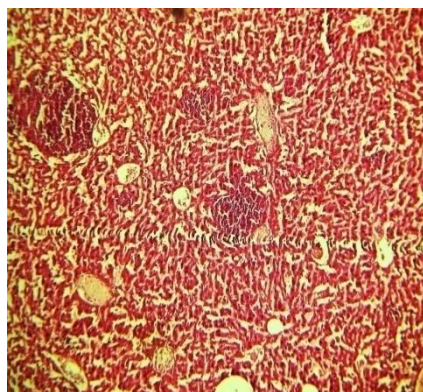


Fig.3. Focal inflammatory cells & congestion in liver of white mice infected with *C. pseudotuberculosis* H&E(X40)

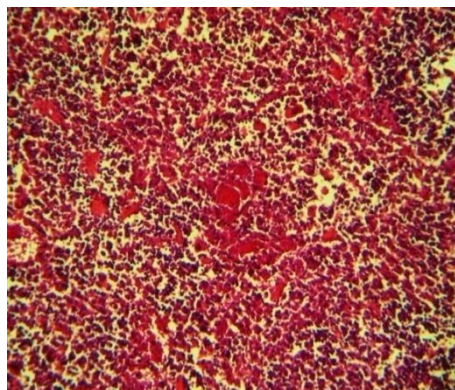


Fig.4. Depletion of lymphocytes, Giant Cells & RBCs in spleen of white mice infected with *C. pseudotuberculosis*
H&E(X40)

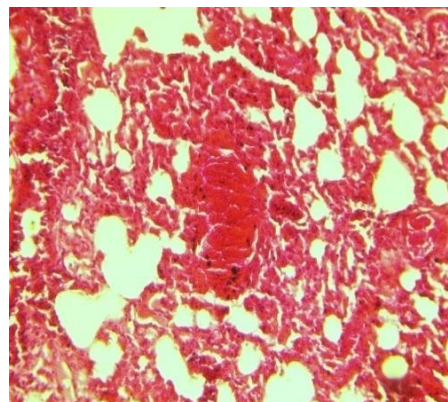


Fig.5. Congestion, thickening in alveolar septa & inflammatory cells in lung of white mice infected with *C. pseudotuberculosis*.
H&E(X40)

Discussion

Identification of *C. pseudotuberculosis* by specific-PCR is useful for several reasons. First, bacteriological tests are time consuming and there are variations in biochemical characteristics of *Corynebacterium* species which have hampered the use of routine bacteriology in differentiating the species belonging to the genus (Hommez and Devriese 1999). Second, it might be useful as a sensitive, specific assay for direct detection and confirmation of CL infection (Cetinkaya *et al.* 2002). In this study PCR was used to identify and confirm 5 isolates of *C. pseudotuberculosis* and all of them were positive by PCR.

The five strains were used to investigate the histopathological response during experimental infection in white mice after ten days from inoculation. The results of this experiment demonstrate that *C. pseudotuberculosis*, isolated from a natural case of caseous lymphadenitis could cause acute disease in white mice.

The five strains differed in their pathogenic involvements in mice, this finding agrees with Suleiman (2006) who have found differences in the pyogenic and cytotoxic effects of different strains, and these differences could be utilized for typing of *C. pseudotuberculosis* strains.

One of the major findings was the focal infiltration of polymorphonuclear cells in liver, lung and spleen, which indicated an acute infection process. As stated by Guilloteau *et al.*, (1991) in *C. Pseudotuberculosis* infection, the transformation of primary foci into pyogranuloma occurred rapidly after inoculation, from day 3 to day 10 post inoculation and relative disappearance of neutrophils associated with an increased participation of macrophages. Pepin *et al.*, (1991) also reported that the development of pyogranuloma is associated with a limited bacterial dissemination. Similar finding was also observed by Junior and Oliveria (2006) in their study of experimental infection of goat's mammary glands by *Corynebacterium pseudotuberculosis* and by Burrell (1978) in his study of experimental induction of caseous lymphadenitis in sheep. In addition, other major finding is the formation of typical pyogranuloma in kidney with central necrosis and a peripheral mantle of mononuclear cells composed of macrophages and lymphocytes, similar to that observed in the natural disease of sheep (Ellis, 1988) or in the rare case of human infections, Blackwell *et al.*, (1974). The pyogranuloma in kidney can be considered as an immunopathological process because of tissue damage and excessive fibrosis associated with the persistence of viable bacteria. The present study is in agreement with those of Pepin *et al.*, (1991), in his study of histopathological changes during early phase of infection in lambs. These results illustrate the formation of granulomatous lesions in chronic bacterial infection.

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