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With 4 figures in the text. *www.sudanjvr net.*

Molecular Characterization of *Corynebacterium pseudotuberculosis* isolated from Sheep and Goats in Western Sudan

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ملخص البحث

استخدمت تقنية تفاعل البلمرة التسلسلى فى تأكيد تشـــخيص 28عزلة لبكتريا وتدية السل الكاذب *Corynebacterium pseudotuberculosis* التى تسبب التهاب العقد اللمفية الجبنى بعد عزلها من ضأن و ماعز فى ولاية جنوب دارفور بغرب السودان. أظهرت نتيجة الرحلان الهلامى للحمض النووى الريبى منزوع الأكسجين لكل هذه العزلات تطابقاً لحجم معدل المورث المستهدف (16S rRNA) لهذه البكتريا. يمكن استخدام هذه التقنية لتشخيص دقيق و سريع لإلتهاب العقد اللمفية الجبنى فـى الضـأن والمـاعز مما قد يساعد فى السيطرة على المرض.

Summary

A PCR was used to examine 28 isolates of *Corynebacterium pseudotuberculosis* isolated from cases of caseous lymphadenitis in sheep and goats in South Darfur State, Western Sudan. Using primers targeting the 16S rRNA gene, electrophoresis of the DNA of the isolates showed homogeneity of this targeted gene. The PCR could be used for rapid and accurate diagnosis of the disease in sheep and goats which may help in it's control.

Introduction

Corynebacterium pseudotuberculosis is the aetiological agent of caseous lymphadenitis (CLA); a chronic debilitating disease of sheep and goats (Ayers 1977). CLA is characterized by the formation of suppurative abscesses in superficial and internal lymph nodes and visceral organs. It occurs worldwide, and adversely affects sheep trade and industry due to carcasses condemnation, wasting effects, poor wool growth, reduction in milk production and, rarely, deaths (Alonso *et al*., 1992; Paton *et al*., 1994). The diagnosis of CLA is mainly based on clinical signs and isolation of the bacterium from abscesses. Identification of *C. pseudotuberculosis* is usually achieved by biochemical tests, but it is often problematic due to extensive variability in biochemical characteristics of the pathogen (Muckle and Gyles, 1982; Songer *et al.*, 1988). Serological tests, such as ELISA (Schreuder *et al.*, 1994; Dercksen *et al.*, 2000), indirect haemoagglutination test (Shigidi, 1979), immunodiffusion test (Burrell, 1980) and the haemolysis inhibition test (Holstad, 1986) have been used for diagnosis of CLA but most of these tests lack sensitivity and specificity. The eradication of CLA is difficult due to the rapid spread of the disease once introduced into a flock. The availability of a sensitive and specific reference test for monitoring the infection is needed.

In the present study a PCR- based assay using a pair of primers specific to *C.pseudotuberculosis/C. ulcerans* that was developed by Çetinkaya *et al* (2002) was used to confirm biochemical identification of 28 isolates of *C.pseudotuberculosis* from sheep and goats in South Darfur State, Western Sudan.

Materials and Methods

Bacterial isolates:

Twenty-eight isolates of *C. pseudotuberculosis*, 22 from sheep and 6 from goats were tested; these isolates were previously identified as *C. pseudotuberculosis* by culturing onto

blood agar microaerophilically under 10% Co_2 tension at 37ºC for 48 hours. The isolates were tested for catalase and urease activities, nitrate reduction and carbohydrates fermentation (glucose, fructose, mannose, maltose, galactose , salicin, trehalose and starch). A wild strain of *C. pseudotuberculosis* CP47 W which was obtained from the Department of Microbiology, Faculty of Veterinary Medicine, University of Khartoum, was used as a reference strain.

DNA extraction:

DNA extraction was made in the same manner for all the 28 *C. pseudotuberculosis* isolates and the reference strain according to Sambrook and Russel (2001). A 20 ml of 48–72 h culture of each isolate was centrifuged at 4 °C and 9000 g for 20 minutes. The supernatant was discarded and 800 µl of TNES buffer (100 mM Tris Hcl (pH 7.6), 40 mM EDTA, 50 mM Nacl, SDS 0.2%) and 10 µl of proteinase K (10 mg/ml) were added to the precipitate which was incubated over night at 37 °C or at 65 °C for 2 hours. An equal volume mixture of phenol/chloroform/isoamyl alcohol was added, mixed and centrifuged at 9000 g for 5 minutes. The upper layer was transferred to a clean tube, before an equal volume of phenol /chloroform/isoamyl alcohol was added. The mixture was centrifuged at 9000 g for 5 minutes and the upper layer was transferred to a clean tube. Two volumes of 95% cold ethanol and 1: 10 of sample volume of 3M Sodium acetate were added and the tubes were incubated over night or at–20°C for 2 hours then centrifuged at 18000 g for 10 minutes. The supernatant was discarded and 2 ml of 70% ethanol were added. The tubes were then centrifuged at 12000 g for 7 minutes and the supernatant was discarded. The previous step was repeated once more before the supernatant was discarded and the pellet was allowed to dry for 15 minutes and 100 µl of TE buffer or deionized water was added and stored at -20° C until used.

Concentration of DNA:

The quantity of DNA for every sample was read by Nano Drop spectrophotometer (ND- 100) and depending on DNA reading either 2.5 or 5 µl was used for the PCR

Primers design:

The primers used in this study were targeting the published 16S rRNA gene sequences of *C. pseudotuberculosis* (GenBank). The selected primers were with a length of approximately 20 bp, with an optimal annealing temperature between 50 and 60° C, and a GC-content between 20 till 80% (Çetinkaya *et al*., 2002). The resulting forward primer was;
ACCGCACTTTAGTGTGTGTG (*E. coli* position 183–203) and the reversed primer had the sequence:
TCTCTACGCCGATCTTGTAT (*E. coli* position 1019–999) and the annealing temperature was 55°C. The specificity of the primers was checked by comparing their sequences to all known sequences in GenBank (Çetinkaya *et al.*, 2002).

PCR mixture:

The total reaction volume was 25 µl which contained: 5µl of 10 × Vi buffer A (Vivantis, DNA Amplification products), 5 µl of 5 mM $MgCl_2$, 1 µl of 10 mM deoxynucleotide triphosphate (dNTPs) (Vivantis, Nucleotides, 0.4 µl of Taq DNA polymerase (Vivantis) conc. 5U/µl, 1 µl of 100 mM of each primer (Vivantis technologies Sdn.Bhd., Malaysia) and 5 or 2.5 µl of template DNA.

PCR conditions:

The PCR was performed in a touchdown thermocycler (Advanced primus 96, Peq lab Biotechnologie, Germany).Amplification was obtained with 30 cycles following an initial denaturating step at 94° C for 5 minutes. Each cycle involved: denaturation at 94° C for 1 minute, annealing at 56° C for 1 minute and synthesis at 72° C for 2 minute.

Detection of PCR product:

Using gel electrophoresis, the amplified products for the isolates with both positive wild strain and negative control were detected by ethidium bromide (0.5 mg/ml) staining after electrophoresis at 60–70 V for 1 h in 1.5% agarose gel. An automated photo - documentation system (Bio. Doc. Analyza, digital) was used for analysis. PCR products with the molecular size of 815 bp were considered indicative for identification of *C. pseudotuberculosis.*

Results

By passing the products of PCR in gel, isolates which were identified morphologically and biochemically as *C. pseudotuberculosis* showed the same designated molecular size (816 bp) for the primer targeting the 16S rRNA gene of *C. pseudotuberculosis*, yet negative control (reaction without template DNA) showed no results. Figures 1, 2, 3 and 4 show results of samples obtained from different isolates.

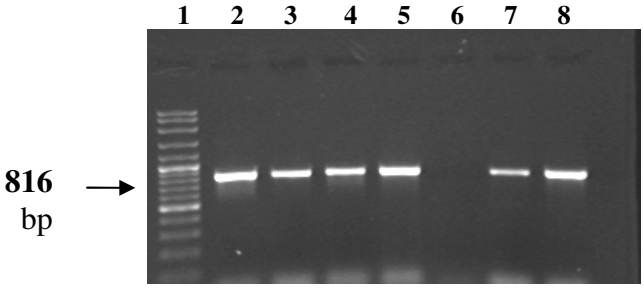

Fig. 1: PCR amplification of *C. pseudotuberculosis* 16S rDNA gene sequences on agarose gel-electrophoresis for strains: CP199N (2), CP201N (3), CP152N (4), CP41N (5), negative control (6), strains: CP275N (7) and CP216N (8), whereas DNA marker ladder is in lane 1.

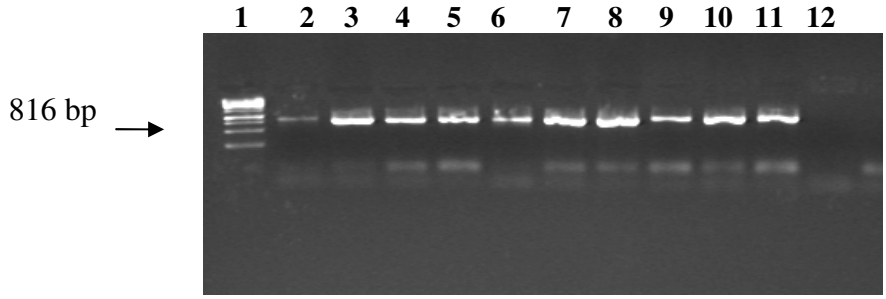

Fig. 2: PCR amplification of *C. pseudotuberculosis* 16S rDNA gene sequences on agarose gel-electrophoresis for strains, CP144N (2), CP148N (3), CP153N (4), CP160N (5), CP162N (6), CP196N (7), CP214N (8), CP221N (9), CP313N (10), CP322N (11) and negative control (12), whereas DNA marker ladder is in lane 1.

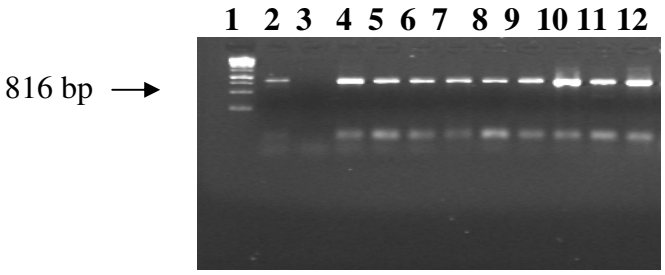

Fig. 3: PCR amplification of *C. pseudotuberculosis* 16S rDNA gene sequences on agarose gel-electrophoresis for strains CP214N (2), negative control (3), CP41N (4), CP64N (5), CP65N (6), CP229N (7), CP203N (8), CP47W (9), CP47W (10), CP187N (11) and CP180N (12) whereas DNA marker ladder is in lane 1.

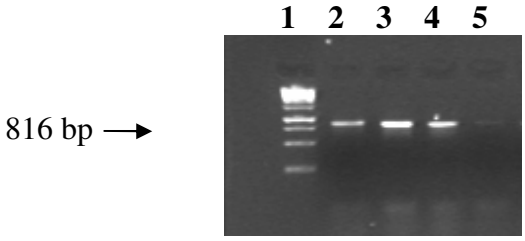

Fig. 4: PCR amplification of *C. pseudotuberculosis* 16S rDNA gene sequences on agarose gel-electrophoresis for strains: CP165N(2), CP230N(3), CP209N (4), negative control (5) whereas DNA marker ladder is in lane 1.

Discussion

PCR targeting the 16S rRNA gene showed positive results for all the isolates identified as *C. pseudotuberculosis* and this is in agreement with Çetinkaya *et al.* (2002), taking into account that it could give the same results with *C.ulcerans.* This degree of similarity of *C. pseudotuberculosis* with *C.ulcerans* was also noted by Barksdale *et al* (1981) who reported that phospholipase D activity has been found in strains exhibiting the biochemical properties characteristic of *C. pseudotuberculosis* or of *C.ulcerans* but in no other species of *Corynebacterium*. Although *C. ulcerans* is known to cause diphtheria-like disease in humans and is also important in cattle; the presence of this species in small ruminants is not recorded in the literature. Some biochemical tests (trehalose, maltotriose), have been reported to distinguish *C. pseudotuberculosis* from *C. ulcerans* (Hommez *et al*., 1999). Recently, a multiplex PCR (mPCR) assay targeting three genes of *C. pseudotuberculosis*: the 16S rRNA gene, rpoB and pld was developed by Pacheco *et al*. (2007). They reported that their mPCR assay was specific enough to differentiate, with a high diagnostic sensitivity (94.6 %) *C. pseudotuberculosis* from *C. ulcerans* Hence, PCR techniques can be used for accurate and rapid diagnosis of CLA which will help in its control.

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