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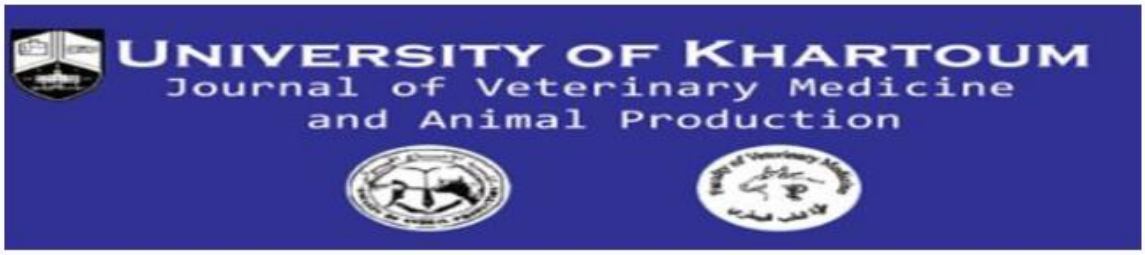

Characterization of *Corynebacterium pseudotuberculosis* from Sudan by Multiplex PCR

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Abstract

A multiplex PCR(mPCR) was used to examine isolates of *Corynebacterium pseudotuberculosis* from cases of caseous lymphadenitis in sheep and goats in South Darfur State, Western Sudan. Using primers targeting the *16S rRNA*, *pld* and *rpoB*genes, electrophoresis of the DNA of the isolates showed homogeneity of these targeted genes. The mPCR could be used for rapid and accurate diagnosis of the disease in sheep and goats which may help in its control.

Keywords: *Corynebacterium pseudotuberculosis,* Caseous lymphadenitis, sheep, Multiplex PCR,

المستخلص

استخدمت تقنية تفاعل البلمرة التسلسلي المتعدد لفحص عزلات لبكتريا وتدية السل الكاذب التي تسبب التهاب العقد اللمفية الجبنى، عزلت من ضأن وماعز في ولاية جنوب دارفور بغرب السودان، استهدفت الدراسة الحمض النووي الريبي منزوع الأكسجين(mPCR)لهذه العزلات وقد وضحت نتيجة الرحلان الهلامي تطابقا في حجم معدل المورثات المستهدفة في البكتريا(*rpoB*؛*16S rRNA* ; *pld*) ، مما يجعل هذه التقنية وسيلة للتشخيص الدقيق والسريع لبكتريا مرض التهاب العقد اللمفية الجبنى في الضأن والماعز وتساعد في السيطرة على المرض.

الكلمات المفتاحية: بكتريا وتدية السل الكاذب، التهاب العقد اللمفية الجبنى، الضان، تفاعل البلمرة التسلسلي المتعدد

Introduction

Caseous lymphadenitis (CLA) is an infectious, transmissible and chronic debilitating disease of sheep and goats (Batey, 1986; Benham *et al*., 2002) caused by *Corynebacterium pseudotuberculosis* (*C. pseudotuberculosis)*. The organism is a small, pleomorphic Gram-positive coccobacillus that ismicroaerophilic (5% CO_2), tolerant to aerophilic conditions and non-sporeforming (Collet *et al*., 1994). The disease is characterized by abscesses that may be superficial, or may affect internal organs (Dorella *et al*., 2006). The Parotid, mandibular, superficial cervical, subiliac, popliteal, or mammary lymph nodes are commonly affected and in some cases internal abscesses are seen in lungs and bronchial lymph nodes. The disease may be asymptomic or may be accompanied with chronic pneumonia, mesenteric lymphadenitis, and pyelonephritis (Paton *et al.*, 1995). 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, which are often variable (Muckle and Gyles, 1982; Songer *et al*., 1988). Serological tests, such as indirect haemoagglutination test (Shigidi, 1979), immunodiffusion test (Burrell, 1980), haemolysis inhibition test (Holstad, 1986) and ELISA (Schreuder *et al*., 1994; Dercksen *et al*., 2000) 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.

Çetinkaya *et al*. (2002) reported that PCR targeting the *16S rRNA* gene showed positive results for all the isolates identified as *C. pseudotuberculosis.* Abdel Wahab and Shigidi (2012) reported similar results using this method. Pacheco *et al*. (2007) developed a multiplex PCR (mPCR) assay targeting three genes of *C. pseudotuberculosis*: the *16S rRNA*, *rpoB* and *pld* genes,he reported that this mPCR assay was specific and with a high degree of sensitivity (94.6 %) to identify *C .pseudotuberculosis.* Therefore, PCR techniques can be used for accurate and rapid diagnosis of CLA which will help in its control.

This study was done to confirm the identification of five isolates from sheep and goats which had been recognized on the basis of their morphological, cultural and biochemical characteristics as *C. pseudotuberculosis*. Confirmation was done by mPCR- based assay using three pairs of primers specific to *C. pseudotuberculosis;* the *16S rRNA*, *rpoB* and *pld* genes (Pacheco *et al*.,2007).

Materials and Methods

Bacteria and DNA isolation:

Five isolates of *C. pseudotuberculosis*; three from sheep and two from goats previously identified as *C. pseudotuberculosis*and designated asCP201N, CP152N, CP41N, CP275Nand CP216Nwere used. A wild strain of *C. pseudotuberculosis* designated as CP47 W, which had been obtained from the Department of Microbiology, Faculty of Veterinary Medicine, University of Khartoum, was used as a reference strain. Distilled water (D.W) used as negative control.

DNA isolation was made in the same manner for all isolates according to the method of Sambrook and Russel (2001). A 20 ml amount of a 48– 72 hours culture in brain heart infusion medium 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 mMTrisHcl (pH 7.6), 40 mM EDTA, 50 mMNacl, SDS 0.2%) and 10 µl of proteinase K (10 mg/ml) were added to the precipitate, which was incubated overnight 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. The tubes were incubated 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 the contents were stored at -20° C until used.

Primers and concentration of DNA:

The oligonucleotide primers used in this study are listed in Table 1. Primers targeting the 16S rRNA, *pld* and *rpoB* genesof *C. pseudotuberculosis* were obtained from Vivent Company, Malaysia. 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.

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 MgCl2, 1 µl of 10 mMdeoxynucleotide 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.

Table 1. List of oligonucleotide primers used in this study

Target gene	Primers	Sequence (5'→3')	Length of PCR products (bp)	Source/reference
16S rRNA gene	16S-F	ACCGCACTTTAGTGTGTGTG	816	Çetinkaya *et al.* (2002)
	16S-R	TCTCTACGCCGATCTTGTAT		
rpoB	C2700F	CGTATGAACATCGGCCAGGT	446	Khamis *et al.* (2004)
	C3130R	TCCATTTCGCCGAAGCGCTG		
pld	PLD-F	ATAAGCGTAAGCAGGGAGCA	203	Pacheco *et al.* (2007)
	PLD-R1	ATCAGCGGTGATTGTCTTCC		
	PLD-R2	ATCAGCGGTGATTGTCTTCCAGG		

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 minutes.

Detection of PCR product:

Using gel electrophoresis, the amplified products for the isolates with both the positive wild strain CP47W and a negative controlD 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. mPCR products with the molecular size of 815, 446 and 203 bp were considered confirmative 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), (446bp) and (203 bp) for the primers targeting the *16 rRNA*, *rpoB* and *pld* genes of *C. pseudotuberculosis* respectively, yet negative control (reaction without template DNA) showed no results. Figure 1 shows results of samples obtained from different isolates.

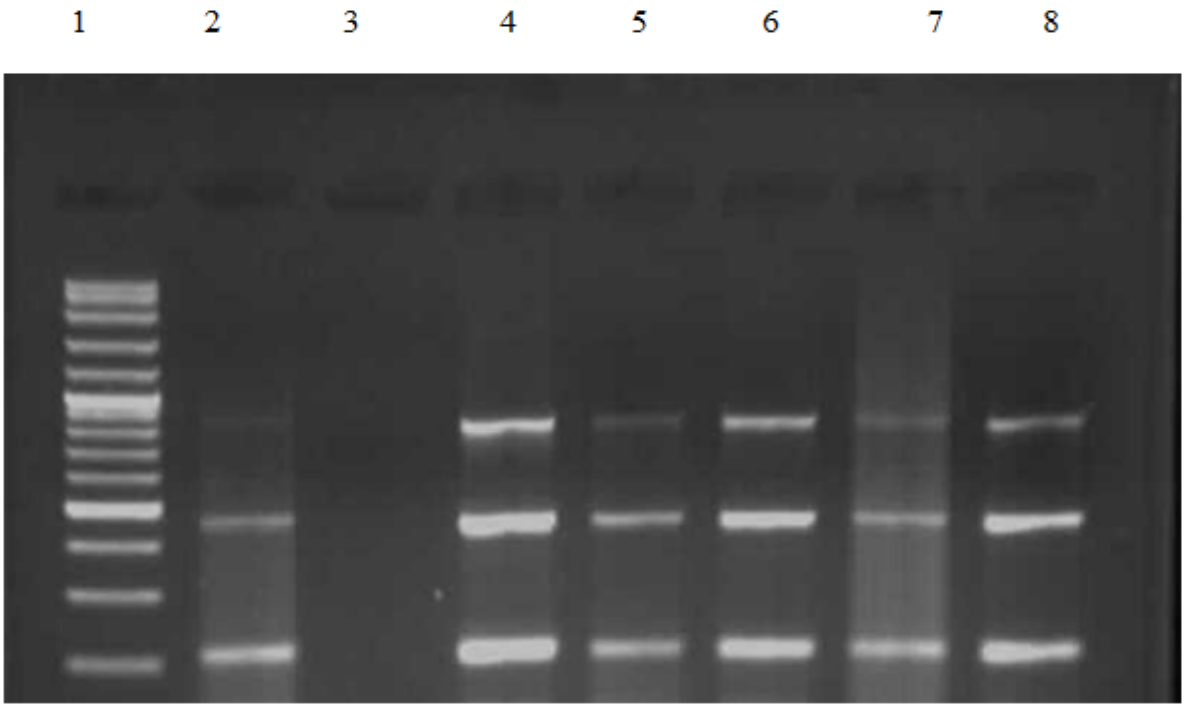

Fig. 1: PCR amplification of C. pseudotuberculosis16S rDNA, rpoB and pldgenes sequences on agarose gel electrophoresis for strains: CP201N (4), CP152N (5), CP41N (6) CP275N (7) and CP216N (8). Whereas DNA marker ladder is in lane 1, CP47W (2) positive control and D.W (3) negative control.

Discussion

Caseous lymphadenitis remains an obstacle for the development of the sheep and goats industry. Due to its high transmission rate, some authors suggest culling of allinfected animals from which the bacterium has been cultured as a means of controlling spread of the disease (Baird, 1997; Williamson, 2001). Çetinkaya *et al*. (2002) stated that as microbiological and biochemical methods are not always conclusive, the development of a rapid and specific diagnostic method is imperative for the control of CLA; therefore, they developed a PCR technique of *16S rRNA* gene-based PCR assay to identify *C. pseudotuberculosis* isolates and to estimate

the prevalence of CLA in the animals studied. Khamis *et al*. (2005) reported that higher proportions (91%) of 168 corynebacterial isolates were positively identified by partial *rpoB* gene determination than by that based on *16S rRNA* gene sequences. Ilhan (2013) found that PCR sensitivity and specificity to culture being 98.76% and 92.42%, respectively using *pld* gene DNA fragment. Amplification of multiple loci in a single reaction through mPCR is a powerful and widely used as a tool for the rapid and specific identification of pathogenic bacteria (Wadowsky *et al*., 1996; Halbert *et al*., 2005; O'Halloran and Cafferkey, 2005; Persson and Olsen, 2005).In order to improve *C. pseudotuberculosis* detection by PCR, a multiplex PCR (mPCR) assay was developed by Pacheco *et al*. (2007) targeting three genes of *C. pseudotuberculosis*: the 16S rRNA, *rpoB* and *pld* genes. They reported that their mPCR assay was specific enough to differentiate with a high diagnostic sensitivity (94.6 %) *C. pseudotuberculosis* from *C. ulcerans.* The mPCR enabled specific identification of *C. pseudotuberculosis* isolates in culture. Hence, our findings are in agreement with that of Pacheco *et al*. (2007) targeting the three genes (Fig.1).

Conclusion

The mPCR due to its high sensitivity and rapidity, could be used as an alternative to traditional bacteriological culture methods for the detection and confirmation of *C. pseudotuberculosis* from naturally infected sheep and goats; this is particularly important in understanding the epidemiology of CLA and the control of the disease.

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