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MOLECULAR SURVEY FOR THE PRESENCE OF *Corynebacterium pseudotuberculosis* FROM SHEEP AND CAMEL LYMPH NODES BY PCR IN IRAN

F. Khamesipour[1], A. Doosti[2], A. Koohi[3] and B. Radojicic[4]

[1]Young Researchers and Elite Club, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran
[2]PhD, Biotechnology Research Centre, Islamic Azad University, Shahrekord Branch, Shahrekord, Iran
[3]School of Veterinary Medicine, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran
[4]University of Belgrade, Faculty of Veterinary Medicine, Bulevar Oslobodjenja 18, Belgrade, Republic of Serbia

ABSTRACT

The carcasses of sheep (500) and camel (150) slaughtered for human consumption as food were examined for the presence of pseudotuberculosis (Caseosus Lymphadenitis abbreviated - CLA) lesions in the Najafabad Slaughterhouse, Isfahan province, Iran, during December 2012-March 2013. Also, a total of 150 camel carcasses were examined. The lymph nodes were forwarded to the laboratory for analysis. Abscessed lymph nodes (prescapular) were taken from 78 carcasses from both (41 sheep and 37 camel). The samples were immediately transported to the Biotechnology Research Centre of Islamic Azad University of Shahrekord Branch laboratory. PCR method was used to isolate *C. pseudotuberculosis* from 11 out of 78 abscessed lymph nodes collected in 78 animals, with 4/37 isolated in camel and 7/41 in the sheep, displays the infections in the different sex categories, but predominant positive cases were in the female animals in both the species.

Key words: Camel, *Corynebacterium pseudotuberculosis*, Iran, PCR, lymph nodes, sheep

Pseudotuberculosis (Caseosus Lymphadenitis abbreviated - CLA) is a chronic disease that affects the lymph nodes of sheep and goats and rarely man. It is caused by *Corynebacterium pseudotuberculosis*. The disease is spread almost all over the world. proven in Europe, North and South America, South Africa, Middle East and New Zealand.

Protein chain reaction (PCR) is better diagnostic technique for infectious diseases caused by fast pus or slow-growing bacetria (Pacheco *et al,* 2007).

The sensitivity of the micro-organism *Corynebacterium pseudotuberculosis* antibiotics tested *in vitro*, is different and depends from region to region (Literak *et al,* 1999; Connor *et al,* 2000; Foley *et al,* 2004). Muckle and Gyles (1982) examined 26 strains isolated from lesions of caseous lymphadenitis in goats. All isolated strains were sensitive to ampicillin, chloramphenicol, lincomycin, gentamicin, tetracycline and penicillin G.

CLA antibiotic therapy was unsuccessful primarily because, after the application thereof, the bacteria remain alive, which is protected by the surrounding thickened abscess capsule (Piontkowski and Shivvers, 1998; Stanford *et al,* 1998; Williamson, 2001). The present study is based on molecular survey for the presence of *Corynebacterium pseudotuberculosis* from sheep and camel lymph nodes by PCR in Iran.

Materials and Methods

Animals and samples

The carcasses of sheep (500) and camel (150), slaughtered for human consumption as food were examined for the presence of CLA lesions in the Najafabad Slaughter house, Isfahan province, Iran, during December 2012-March 2013. Abscessed lymph nodes (prescapular) were taken from 78 carcasses from both animals (41 sheep and 37 camel) under strict aseptic precautions, with a sterile scalpel and placed in individual sterile containers. The samples were immediately transported to the Biotechnology Research Centre of Islamic Azad University of Shahrekord Branch laboratory and were processed for culture, and stored at -20°C for further use.

Polymerase Chain Reaction (PCR) Assay

To avoid possible contamination, the extraction, amplification and electrophoresis stages of the PCR were performed in a separate cabinet. All plasticware used were DNAse and RNAse free and disposable

SEND REPRINT REQUEST TO F. KHAMESIPOUR email: Dr_Faham@yahoo.com and F.Khamesipour@iaushk.ac.ir

were not re-used throughout the experiment. Different sets of micropipettes were used at each step of sample processing, DNA extraction, PCR-mix preparation and electrophoresis.

DNA extraction from lymph nodes

Immediately prior to DNA extraction, samples were thawed at room temperature and any fat, pus or caseous mass present was removed using a sterile scalpel. A 4g portion of lymph node sample (pooled prescapular lymph nodes) was placed in a stomacherTM (Seward Ltd., West Sussex, UK) with 8 mL of sterile phosphate-buffered saline (PBS, pH 7.2) and homogenised for 10 minutes. An aliquot of the homogenate (300 µL) was placed in a microtube for DNA extraction.

Next, the same volume of lysis solution (10 mM Tris-HCl, 1% SDS, 100 mM NaCl, 2% Triton-X100, pH 8.0) and 15 mL of proteinase K (20 mg/ mL) (Qiagen, Hilden, Germany) were added to the samples, and the contents were mixed thoroughly. Following 1 hour of boiling, saturated phenol (liquid phenol containing 0.1% 8-hydroxyquinoline, stabilised with 100 mM Tris-HCl, pH 8.0, and 0.2% 2-mercaptoethanol, 300 mL) was added; the contents were mixed vigorously for 5 minutes and then centrifuged at 11,600 g for 10 minutes at room temperature. An equal volume of chloroform-isoamyl alcohol (24:1) (Applichem, Darmstadt, Germany) was added to the aqueous layer and after mixing thoroughly for 5 minutes, tubes were centrifuged as before. Then, 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of 95% ethanol were added to the upper layer and mixed thoroughly. After incubation overnight at -20°C to precipitate the DNA and centrifugation at 13,000 g for 10 minutes at 0°C, the supernatants were discarded and the pellets were washed sequentially with 95% and 70% ethanol.

The samples were then vortexed and centrifuged at 11,600 g for 5 minutes at 4°C. The pellets were dried and suspended again in 50 µL of TE (Tris-EDTA) buffer (Applichem). In addition, for standardisation of the extraction method, a commercial DNA-extraction kit (Cinnagen, Tehran, Iran) was also used according to the manufacturer's recommendations. For this purpose, 25 mg of the pooled lymph tissue samples were used for the initial extraction material. The total DNA was measured at 260 nm optical density according to the method described by Sambrook and Russell. Samples were stored at -20°C until used as templates for amplification.

Primers, amplification conditions and agarose gel electrophoresis

Polymerase chain reaction was performed on the oligonucleotide primers used for PCR were from the pld gene of *C. pseudotuberculosis* to amplify a 203 base pair (bp) using the following set of primers: PLD-F: 5'- ATA AGC GTA AGC AGG GAG CA -3' and PLD-R2: 5' ATC AGC GGT GAT TGT CTT CCA GG -3' (Pacheco *et al,* 2007). The procedure for amplification of the DNA was done as reported previously (Pacheco *et al,* 2007).

Amplification-reaction mixtures were prepared in volumes of 50 µL containing 5 µL of 10X PCR master mix (Fermentas, Vilnius, Lithuania), 5 µl of 25 mM $MgCl_2$, 0.2 µL of 10 mM dNTP mixture (Fermentas), 2 U of Taq DNA polymerase (Fermentas), 1 µmol of 25 mM each primer, 0.1% Triton-X100 (Applichem) and 5 µl of template. Bovine serum albumin (BSA, 10 g/L, 1 mL) (Sigma-Aldrich Corp., St. Louis, MO, USA) in 0.85% NaCl was added as an enhancer to each PCR reaction mixture. PCR was performed in a DNA thermocycler (Eppendorf, Hamburg, Germany) and amplifications were performed using the following protocol: after an initial denaturation at 94°C for 5 minutes, the next 35 cycles were 94°C for 1 minute (denaturation), 56°C for 1 minute (annealing), and 72°C for 2 minutes (extension). The final extension was at 75°C for 5 minutes. The negative control contained sterile water.

The amplified products were analysed by electrophoresis on a 2% (w/v) agarose gel run at 80-90 volts for 1.5-2 hours and then stained with ethidium bromide (0.3 mg/L). Amplified products were images obtained in UVI doc gel documentation systems (Uvitec, UK). The PCR products were identified by 100 bp DNA size marker (Fermentas, Germany) and PCR products with a molecular size of 203 bp were considered positive for *C. pseudotuberculosis*.

Statistical analysis

Analysis of data was performed by the SPSS version 17.0 computer software (SPSS, Chicago, IL).

Results and Discussion

The distribution of *C. pseudotuberculosis* in camels and is presented in table 1. It also displays the infections in the different sex categories. The presence was more in the sheep (about 18%) as compared to camel is 11% cases. In 7 positive cases of sheep, 5 female animals (71.43%) and in 4 positive camel of 3 (75%) female animals. Predominant positive cases are in female animals in both the investigated breeds.

The findings of the present study suggested that all clinical cases indistinguishable from CLA could be confirmed by the bacterial culture and PCR. The PCR assay was rapid, specific and as significant as bacterial culture in detecting bacteria directly in the clinical pus samples. The PCR assay offers advantages over bacteriological culture in terms of reduced time and its ability to also detect non-viable bacteria in pus sample. Inclusion of 3 specific genes as target confers specificity and sensitivity to PCR for detection of *C. pseudotuberculosis* directly from the clinical samples as well for the confirmation of *C. pseudotuberculosis* isolates. The assay can be applied further to detect *C. pseudotuberculosis* from other form of clinical sample from other animal disease conditions and even from human's cases (Kumar *et al,* 2013).

Table 1. Molecular frequency of *C. pseudotuberculosis* in Iran based on sex of sheep and camel.

Animal	Total Prevalence		Prevalence by sex (%)	
	n	Positive samples	(n) Female (%)	(n) Male (%)
Sheep	41	7 (17.07%)	5 (71.43%)	2 (28.57%)
Camel	37	4 (10.81%)	3 (75%)	1 (25%)
Total	78	11(14.10%)	8 (72.73%)	3 (27.27%)

n=Total number of animals

Corynebacterium pseudotuberculosis was isolated from 55.1% (81/147) of abscessed lymph nodes collected in 147 animals, 39.5% with (32/81) isolated in winter and 60.4% (49/81) in the summer. In addition, 61.7% (50/81) isolates were cultured from prescapular lymph nodes, 14.8% (12/81) from mediastinal lymph nodes, and 23.4% (19/81) were present in both lymph nodes. *Corynebacterium pseudotuberculosis* was not isolated from the remaining 44.8% (66/147) samples, but 50.0% (33/66) of these were positive for *Staphylococcus* spp., 25.8% (17/66) for *Streptococcus* spp., and 21.2% (14/66) for *Escherichia coli*. Infection with pseudotuberculosis in sheep, and camel in Iran is viewed from Veterinary Public Health, thus represents a potential threat to human health (Ilhan, 2013).

From the 468 sheep carcasses randomly selected, 197 cases were suspected to have CLA with signs of prominent enlargement in one of the lymph nodes. In 59 cases (12.60%) pure *C. pseudotuberculosis* was isolated. In 94 (20.8%) cases histopathological study represented pathognomonic signs of CLA (onion ring signs). The average age of the population was 2.92 years, and there was an increase in the frequency of CLA with age (P<0.05). From 59 cases that isolated *C. pseudotuberculosis*, 40 were male and 19 (4.05%)

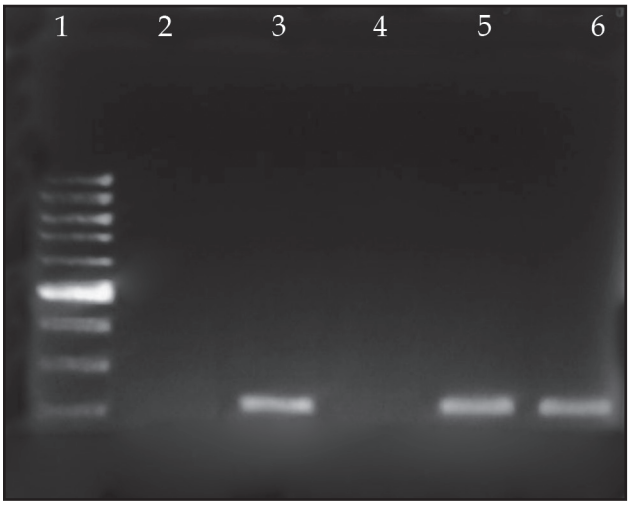

Fig 1. *Corynebacterium pseudotuberculosis* polymerase chain reaction (PCR) assay products obtained from camel and sheep lymph tissue samples separated on a 2% (w/v) agarose gel. Lane 1: Molecular marker; Lane 2: control negative (sterile water); Lane 3, 5 and 6: PCR positive samples (203 bp).

were female, this did not represent a statistical difference (P>0.05). One hundred twelve (56.9%) cases did not result in any isolated microorganisms. In 12 (2.56%) cases *Staphylococcus aureus*, in 4 (0.85%) cases *Bacillus*, and in 3 (0.64%) cases both pure *C. pseudotuberculosis* and *Staphylococcus aureus* were isolated in microbiology. Macroscopic examination of prescapular lymph nodes represented 36 cases with lamellate exudates, but the microscopic study showed that 66 (61.68%) of the samples had CLA signs, resulting in the highest frequency of CLA; a statistical difference with infection compared with other lymph nodes (P<0.05). In all cases studied the microscopic examination gave a more precise diagnosis compared with the macroscopic examination (P<0.05). Thirty-seven cases out of 197 had both microbiology and histopathology results that identified CLA (Rezazadeh Zavoshti *et al,* 2012).

The availability of a *C. pseudotuberculosis*-specific PCR might be useful on several occasions. First, it might be used as an aid in the identification of cultured organisms, since final biochemical identification may not always be straight forward. Second, it might be useful as a sensitive, specific assay for direct detection of CLA infection and as such be used as an aid in vaccination studies. This abattoir-based study was carried out to estimate the prevalence of CLA infection in small ruminants and 2.2%were found positive by both culture and PCR. The proportions of CLA infection were 3.5 and 1.1% in sheep and goats, respectively, when

culture results—indicating *Corynebacterium* spp. Isolation were considered; and 3.4% for sheep and 1.1% for goats, when the PCR results indicating identification as *C. pseudotuberculosis* were accounted. The difference between prevalence in sheep and goats was statistically significant, which suggests that CL is more widespread in sheep than in goats (Cetinkaya *et al*, 2002).

The consequences of this disease are of great economic importance. The bacteria can enter the body and by the oral route, whether in the primary contamination of food, water, or waves (Baird, 2001).

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