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Bacteriological and Molecular Studies of Ovine Caseouslymphadenitis in Iraq

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Abstract: *on clinical examination of 1020 adult sheep from different flocks in and out of Baghdad. The superficial lymph nodes which were showed lesion was 82 (2.55%). The Corynebacterium pseudotuberculosis causes ofCaseous Lymphadenitis CLA was isolated from 26 (8.04%).Parotid lymph node (32.86%) was mostly infected. Genomic DNA was extracted then singleplexPolymerase Chain Reaction (PCR) was used for detection of genes fragments of 16S rRNA, pld and rpoB to confirm the Corynebacterium pseudotuberculosisisolatated from bacterial culture.*

Keywords: *Caseous Lymphadenitis, PCR, Sheep, Corynebacterium pseudotuberculosis*

I. Introduction

Caseous lymphadenitis (CLA) is a chronic infectious disease of small ruminant (sheep and goats) caused by the bacterium *Corynebacterium pseudotuberculosis* was formerly known as *Corynebacterium ovis* (Patton, 2010). CLA is characterized by abscess formation in one or more lymph nodes associated with granulomatous inflammation that lead to enlargement of lymph nodes and chronic abscessation in lymph nodes and internal organs, loss of hair and finally rapture of abscess and pus discharge (Braid and Fontaine 2007). Caseous abscess may occur in internal visceral as well as superficial lymph nodes (Cetinkaya, *et. al*., 2002). The disease is a worldwide distribution and formed in USA, Newzelend, Europe, Austrilia, Africa and Asia (Guimares, *et al* 2011b). CLA was reported in goats in Mosul, Iraq (AL-Sadi, *et. al*., 1998). In Baghdad *C. ovis* was isolated from the horses by Amber who was referred to the one isolation from sheep in his MSc thesis submitted to college of veterinary Medicine, University of Baghdad (Amber, 1989) The disease found in the major sheep and goat production all over the worlds which was cause significant economic losses duo to culling of affected animals, decrees in reproductive efficiency in meat, wool and milk production, carcass and skin condemnation duo to abscesses and production losses because of internal abscesses that may be predominant (Arsenault, *et. al*., 2003).

II. Materials And Methods

Pus collection

Eighty tow pus samples were collected from 1020 clinically examined sheep from different sheep flocks. The pus collections were done either directly using sterile swab from opened abscessed lymph node or by using sterile disposable syringe from non-opened lymph nodes. All animals in visited farms examined grossly for any external nodules especially enlarged one which was palpated as in table (1). The enlarged lymph node of affected sheep was clipped and shaved in small area and cleaned with methanol 70% to avoid environmental contamination during aspiration. Odorless, creamy to caseated pus was aspirated aseptically from enlarged superficial lymph nodes using disposable syringe and needle (gauge 18).The pus sample pushed in disposable tube contained commercial transport media .then all samples were transported to the laboratory under sterile and cooled condition until the required tests were done.

Table (1) Distribution of superficial lymph nodes lesions among clinical examined sheep

Prescapular LN	Mandibular LN	Parotid LN	Total
7	5	70	82

Culture of samples

The samples collected from enlarged lymph nodes and swabs from pus were cultured in tryptic soya broth and on tryptic agar, incubated at 37^0C for 24-48 hours under both aerobic and anaerobic conditions. The incubated broth was subculturedon tryptic soya agar, blood agar, MacConkey agar. Suspected colonies subcultured on Tellurite blood agar and Colombia blood agar.

Molecular characterization of *C. pseudotuberculosis* by PCR

DNA extraction

The genomic DNA extraction of *C. pseudotuberculosis* was done using Presto Mini g DNA Bacterial Kit (Geneaid USA) according to kit instruction. The purity and concentration of extracted DNA was measured using nanodrop spectrophotometer. The eluted DNA extracted was loaded by 1% agarose gel electrophoresis.

Primers used

Three primers in this study were obtained from Integrated and Technologies (IDT), USA.These primers were used to detect *C. pseudotuberculosis*at specie level. These primers were selected according to the previously published work .16S rRNA, rpoB and pld genes were used by (Paheco, *et. al.,* 2007) as showed in table (2)

Table (2) Primers used in the study

gene	Primers	Sequence	bp
16S rRNA	16S-F	ACCGCACTTTAGTGTGTGTG	816
	16S-R	TCTCTACGCCGATCTTGTAT	
rpoB	C2700-F	CGTATGAACATCGGCCAGGT	446
	C3130-R	TCCATTTCGCCGAAGCGCTG	
pld	pld-F	ATAAGCGTAAGCAGGGAGCA	203
	pld-R1	ATCAGCGGTGATTGTCTTCC	

Detection of genes by using conventional PCR

For detecting 16s rRNA, rpoB, and pld genes of *C. pseudotuberculosis* by PCR, the PCR amplification mixture which was used for detection the gene includes master mix, 1 µl of template DNA, 1 µl of each forwarded and reversed primers and 17 µl of nuclease free water to complete the amplification mixture to 20µl. The PCR tubes containing an amplification mixture were transferred to thermocycleras described by (Paheco, *et. al.,* 2007).

III. Results

Bacterial isolation

On clinical examination of 1020 adult sheep from different flocks in and out of Baghdad, 82 (8.04%) sheep were found to have lesion of superficial lymph nodes at different stages of abscessation and enlargement.. Out of 82 pus samples 26 (31.7%) were found to be positive on culture examination and *C. pseudotuberculosis* was isolated giving a result of overall percentage of 2.55 of clinically examined animals as in Table (3). All pus samples were collected just from ewes only and no clinically affected rams were observed during this study. All animals showed no more than one lymph node affected.

Table (3) The percentage of clinical examination of sheep and bacterial culture results.

Test	Tested No.	Positive No.	percentage	Overall %
Clinical exam.	1020	82	8.04	8.04
Bacterial culture	82	26	31.7	2.55

The clear characteristic presences of slightly soft or palpable hard subcutaneous enlargement in the position of superficial lymph nodes were suspected the CLA cases. The lymph nodes affected were prescapular, submandibular, and parotid which were painless on palpation. Two (28.57%) out of 7 prescapular, one (20%) out of 5 submandibular, and 23 (32.86%) out of 70 parotid lymph nodes lesions were found positive on microbiological examination as in Table (4).

Table (4) The distribution of lymph nodes clinically affected and diagnostic cultural results.

Lymph nodes	No. of affected	No. of +ve culture	Positive %
Prescapular	7	2	28.57
Submandibular	5	1	20
Parotid	70	23	32.86
Total	82	26	31.7

The large swelling affected nodes varied in size and diameter measuring about 2.5 cm x 3.5 cm x 7 cm. All the cases showed abscesses with caseous, cream-white, or pale green pus with variable consistency from soft to pasty as in figure (1).

Figure (1) Greenish caseated pus in submandibular lymph node

No systemic reaction recorded in any of the affected animal except one ewe was emaciated, recumbent suffered from what is known as thin ewe syndrome.

Identification of isolates

Corynebacterium pseudotuberculosis colonies were small whitish and surrounded by a narrow zone of complete hemolysis on blood agar (may not be evident for up to 3 days) The colony became dry, crumbly and cream colored after several days (Markey, *et. al.,* 2013). The bacterial isolates were positive on catalase, urease, and Methyl Red test (MR) and characterized by the fermentation of carbohydrate such as glucose, galactose, maltose, and mannose. The nitrate reduction, gelatin hydrolysis, and oxidase were negative on tests. The CAMP test was done and demonstrated the synergistic hemolytic effect between *C. pseudotuberculosis* and *R. equi*.

Detection of*C. pseudotuberculosis* genes

The PCR amplification results which were accomplished on the DNA extracted including all isolates confirmed by the electrophoresis analysis. The estimation of DNA weight on gel electrophoresis had been done by comparing with DNA marker (ladder).

A total of 26 isolates *of C. pseudotuberculosis* identified microbiologically were analyzed using specific PCR for detection *C. pseudotuberculosis* species specific 16S rRNA gene fragments. The results of DNA amplification of *C, pseudotuberculosis* revealed that all samples (100%) were positive. A PCR amplified DNA fragment of 203 bp specific for the *C. pseudotuberculosis*pldgene was applied. The confirmation results of the 26 isolates from suspected CLA cases that recovered from enlarged lymph nodes by PCR to detect the presence of specific virulence pld gene revealed 22 (84.61%) isolates out of 26 were positive to this gene.The 446 bp fragment of rpoB gene was amplified using singleplex PCR using specific pair primers C2700F and C3130R. Twenty five (96%) out of 26 DNA samples that tested carried the rpoB gene as shown in figures 3, 4, 5.

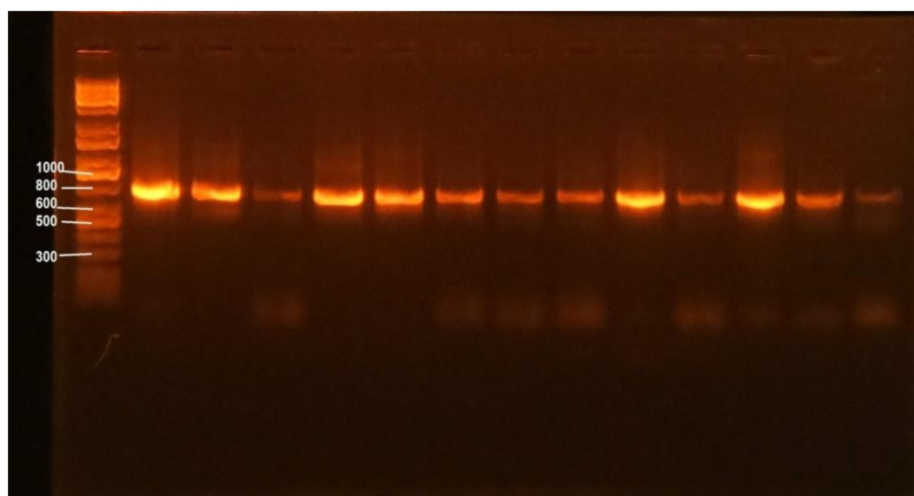

Figure (3) PCR product on 1.5% agarose gel electrophoresis showed amplification of 816 bp fragment of 16S rRNA gene. Lane M represent DNA ladder

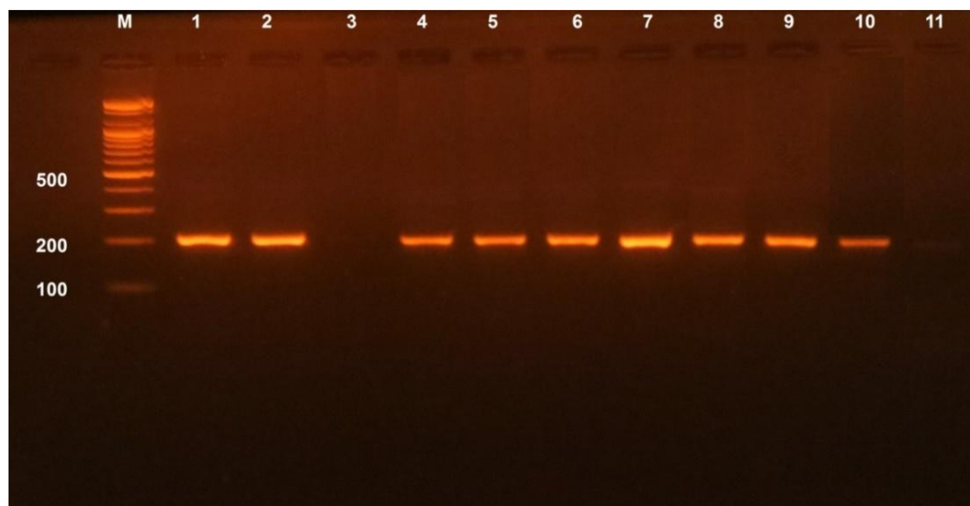

Figure (4) PCR product on 2% agarose gel electrophoresis showed amplification of 203 bp fragment of pld gene. Lane M represent DNA ladder.

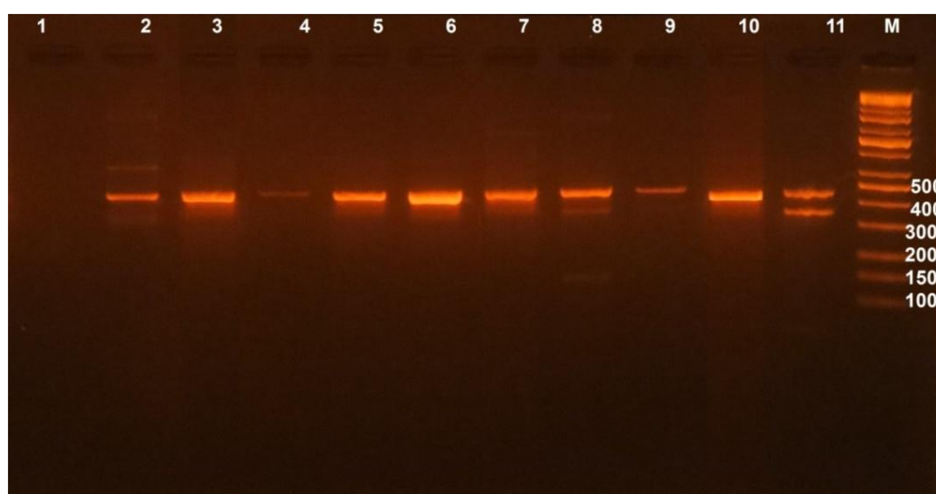

Figure (5) PCR product on 2% agarose gel electrophoresis showed amplification of 446 bp fragment of rpoB gene. Lane M represent DNA ladder

IV. Discussion

The data of CLA incidence in sheep flocks in Iraq have not been recorded yet. Caseous lymphadenitis in sheep in Iraq is a disease which was not studied until now. The percentage of positive number of clinical examination in this study was 8.04% and this result agreed with the results done by (Voigt, *et al* 2012; Chikhaoui and Khoudja, 2013) that 8.6% and 5.7% was recorded. While (Kumar, *et al* 2013) in adult sheep (2.31%) were found to have infection in the superficial lymph nodes. The same results were done on bacteriological isolation; out of 82 pus samples 26 of them were recovered *C. pseudotuberculosis* as in line with (Al-Gaabary, *et al*., 2009; Hasssan, *et al* 2011; and Voigt, *et. al*., 2012). An overall giving proportion of (2.55%) of animals examined clinically recorded in this study was very appropriate to (Kumar, *et al*., 2013) study observed.

Regarding the gender, the prevalence of CLA in many studies were recorded significantly higher in female than male sheep (Al-Gaabary, *et. al.*, 2009; Asaad, 2012; and Chikhaoui, and khoudja, 2013). Concerning the distribution of CLA lesions in superficial lymph nodes in clinically affected sheep, the most frequently were parotid lymph nodes and this fact was reported by (Malone, *et al*., 2006; and Al-Gaabary, *et. al*., 2009;). The other affected lymph nodes were prescapuler and submandibular as cleared by (Asaad, 2012; and Kumar, *et al*. 2013). Thin ewe syndrome associated (a chronic emaciation of ewes despite a good appetite and in absence of parasitosis or specific clinical signs) with gradual emaciation, weakness, and loss weight was observed in one ewe affected with CLA have been already studied in several reports (Renshaw, *et. al.*, 1979; Radostits, *et. al*., 2007, and Guimaraes, *et. al*., 2011b).

PCR is an accurate, efficient, and highly sensitive method that can rapidly lead to the identification of pathogen in material collected directly from lesions as well as isolates in culture (Pacheco, *et. al*., 2007).

Corynebacterium pseudotuberculosis strains were positive for the amplification of a 16S rRNA gene of 815 bp fragment using oligonucleotides specific for *C. pseudotuberculosis*biovarovis in 100% of tested isolates. The 16S rRNA gene is the gene of choice for most microbial taxonomy studies (Khamis, *et. al*., 2005) Of 96 isolates, amplification of 815 bp fragment was obtained in 93 isolates (Cetinkaya, *et. al*., 2002). The 16S rRNA gene sequence analysis a reliable tool for identifying *Corynebacterium* strains (Khamis, *et. al*., 2004). The presence of 16S rRNA gene fragment to Corynebacterium species were determined in 11% of isolates using simplex specific PCR analysis (Dlamini, and Ateba, 2014)

PCR amplified DNA fragment of 203 bp specific for the pld gene of *C, pseudotuberculosis* was evident in 84.61% of culture isolates. The sensitivity (frequency of true positive samples among positive cultures) of PCR related to the bacteriological culture was 84.61% and this agreed with (Venezia, *et. al*., 2012; and Ilhan, 2013). The pld gene was found in most of the clinical isolates which is in agreement with (Cetinkaya, *et. al*., 2002; Pacheco, *et. al*., 2007; and Aquino, *et. al*., 2013)). Comparing the proteomes of two strains demonstrated that the PLD was exposed only in virulent isolates (Pacheco, *et. al.*, 2011). The pld gene encoding exotoxin PLD which is sphingomyelinase implicated in the virulence of *C. pseudotuberculosis*. Reduces the virulence of *C. pseudotuberculosis* isolates and prevention of CLA development was done by attenuation of pld gene (McNamara, *et. al*., 1994; and Tachedjian, *et. al*., 1995)).

The 446 bp internal fragment of rpoB gene is the RNA polymerase ß-subunit gene which currently is used for the study of phylogenetic relationships in the genera *Corynebacterium* and *Mycobacterium* (Dorella, *et. al*., 2006)

Amplification of multiple loci in a single reaction through multiplex PCR (mPCR) is currently a powerful and widely used tool for rapid and specific identification of pathogenic bacteria (Dorella, *et al*., 2006; and Paccheo, *et al.,* 2007) The mPCR was identified all *C. pseudotuberculosis* strains yielding at least three amplicons 816 bp corresponding to 16S rRNA, 446 bp corresponding to rpoB, and 203 bp corresponding to pld (Pacheco, *et. al*., 2007; and Torres, *et. al*., 2013).

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