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Bacteriological and Clinicopathological Studies of *Corynebacterium pseudotuberculosis* Isolated from Caseous Lymphadenitis in Sheep

Heba E. Farhan[1*], Fatma M. Youssef[2], Mohamed E. Helal[3]

[1]Bacteriology Department, Animal Health Research Institute (AHRI), Agriculture Research Center (ARC), 12618, Giza, Egypt.

[2]Clinical Pathology Department, Animal Health Research Institute (AHRI), Agriculture Research Center (ARC), 41511, Ismailia. Egypt.

[3]Biochemistry Department, Animal Health Research Institute (AHRI), Agriculture Research Center (ARC), 41511 Ismailia. Egypt.

***Correspondence**
Corresponding author: Heba E. Farhan
E-mail address: dr.hebaemam@yahoo.com

Abstract

Caseous lymphadenitis (CLA) is a chronic, contagious disease that affects sheep and is brought on by *Corynebacterium pseudotuberculosis* (*C. pseudotuberculosis*). The development of abscesses in the peripheral lymph nodes and widespread infections frequently characterizes it. The purpose of this study was to look into the prevalence of CLA in sheep, the virulence gene, drug resistance genes, and the effect of *C. pseudotuberculosis* on hematological and biochemical parameters, as well as the cellular immunity of diseased sheep. Pus and blood samples were collected aseptically from 100 sheep suffering from abscess formation. The *C. pseudotuberculosis* strain isolates were found in a percentage of 14%. Pseudotuberculosis antibiotic sensitivity revealed a higher resistance to β-lactam antibiotics, aminoglycosides, and lincosamides. Out of the 14 isolates of *C. pseudotuberculosis*, seven isolates were selected for investigation of the presence of *C. pseudotuberculosis*. All of the *C. pseudotuberculosis* isolates tested positive for 16S rRNA, RNA polymerase-subunit gene (*rpoB*) genes, β-lactam (*bla*), and integrons (int). Six isolates were positive for the phospholipase D (*Pld*) gene at a percentage of 85%. On the other hand, the aminoglycosides (*aadA2*) gene and the sigma factor E (sigE) gene were detected in percentages of 57% and 28.57%, respectively. Red blood cell count, hemoglobin (Hb), packed cell volume, mean corpuscular volume, mean corpuscular Hb concentration, white blood cell count, neutrophils, lymphocytes, monocytes, eosinophils, basophils, globulin, and total plasma proteins all showed significant changes. Similarly, the results of the biochemical analysis revealed significant changes in liver and kidney functions, as well as calcium concentration. However, there were no significant changes in potassium (K^+). It concluded that *C. pseudotuberculosis* harms the sheep's health in general reflected by all the changes recorded in the hemogram, leukogram, and blood chemistry.

KEYWORDS
C. pseudotuberculosis, *rpoB*, *bla*, int *Pld* gene, *aadA2* gene, sigE

INTRODUCTION

Small ruminant producers in many countries have suffered major financial losses as a result of an outbreak of a highly contagious chronic pyogenic disease that affects sheep and goats (Dorella *et al*., 2006; Guimarães *et al*., 2011; Abdulrahman, 2021; Burmayan and Brundage, 2021). *Corynebacterium pseudotuberculosis* (*C. pseudotuberculosis*) was first isolated by Preisz and Guinand from the kidney abscesses of sheep in 1891 (Domenis *et al*., 2018). *C. pseudotuberculosis* is a Gram-positive bacterium widely known for being facultative intracellular and proliferating within macrophages (Dorella *et al*., 2006), this condition appears as Caseous lymphadenitis illness (Fontaine and Baird 2008; Windsor 2011). Caseous lymphadenitis (CLA) is caused by *C. pseudotuberculosis* and is considered one of the most serious infectious diseases in small ruminants with poor efficacy of treatment (Torky *et al*., 2023). This disease most commonly infects small ruminants such as goats and sheep, causing significant economic losses in this field (Costa *et al*., 2022). A *C. pseudotuberculosis* infection is characterized by the formation of abscesses in superficial lymph nodes (such as parotid and submandibular lymph nodes) or internally (most commonly in lung and mediastinal lymph nodes), causing an inflammatory complex in which inflammatory cytokines are significantly upregulated (Williamson, 2001). Caseous lymphadenitis (CLA), a chronic pyogranulomatous disease characterized by abscess formation in peripheral lymph nodes (external form) and visceral organs (internal form), is primarily caused by *C. pseudotuberculosis* in both sheep and goats, though the external form is more frequently found in goats than the internal form is in sheep (Williamson, 2001; Baird and Fontaine, 2007; De Oliveira Zamprogna *et al*., 2021). After an injury from shearing, tail docking, tagging, castration, or other environmental risks that may produce cutaneous trauma, skin trauma tail docking, tagging, castration, or other environmental risks that may produce cutaneous trauma, skin trauma represents the pathogen's main entry point into the body. The disease can also spread through eating and direct contact with an aerosol, and it has the potential to infect wounds and mucosal membranes. Because *C. pseudotuberculosis* can survive in the environment for several months, farms or flocks may be more vulnerable to it spreading and persisting there. (Williamson, 2001; Dorella *et al*., 2006; De Oliveira Zamprogna *et al*., 2021). CLA is spread from

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animal to animal primarily through direct contact with bacteria excreted from the abscesses caused by *C. pseudotuberculosis* and through contamination of the environment (Underwood *et al*., 2015). Therefore, it is very difficult to control the spread of the disease between animals after its first introduction into a flock and makes its eradication from an infected herd or area seem to be a pipe dream due to its poor response to treatment, ability to stay in the environment, and difficulty recognizing sub-clinically infected animals (Soares *et al*., 2013; Abd El Tawab *et al*., 2019). CLA decreases production and productivity via lowering the milk and meat production and reduces the value of the hide, and in severe cases, fatality may arise of the infected animal itself resulting in financial loss and even rendering the sheep and goats rearing unprofitable (Junior *et al*., 2006; Ribeiro *et al*.,2013). Extraordinarily, CLA has a long incubation period ranging between 3 and 20 weeks. However, shorter incubation periods have been reported (Mahmood *et al*., 2015) during which only a few animals may develop distinct clinical signs such as fever with some other changes in their vitals such as heart and respiratory rates, inappetence and decreased food consumption and alteration in the general health condition. Oddly, CLA has no significant changes in hemogram in goats challenged with *C. pseudotuberculosis*, but toll reflected significantly on the leukogram between challenged groups at different sampling times (Junior *et al*., 2006; Paton, 2010). A definite diagnosis requires a laboratory bacteriological examination of the pus and identification of *C. pseudotuberculosis* via microscopic examination, culture characteristics, biochemical reactions, serological identification by ELISA, and molecular identifications using PCR and sequencing (Baird and Fontaine, 2007; Torky *et al*., 2023). Treatment and control measures include surgical treatment of abscesses and culling of severely infected animals, proper antibiotic treatment based on results of antibiotic susceptibility testing, vaccination, and selection of resistant breeds (Baird and Fontaine, 2007; Torky *et al*., 2023). Sheep experimentally challenged with *C. pseudotuberculosis* showed changes in the plasma proteins and the hemogram (Jeber *et al*., 2016). Additionally, its zoonotic significance gained attention since clinical cases of human lymphadenitis were found to be brought on by consuming raw milk from sick sheep or by coming into close contact with people who had cracked skin and external open abscesses in animals (Abd El Tawab *et al*., 2019). The incidence of the disease in small ruminants in Egypt's various regions. Distinct geographic locations in Egypt still have different disease prevalence rates among small ruminants. According to Oreiby *et al*. (2014), El-Gharbia Governorate had a prevalence of 6.7%, and the prevalence of *C. pseudotuberculosis* was 41.3% and 13.9% in Menoufia Governorate (Algammal, 2016) and Beni-Suef Governorate (Abd El Tawab *et al*., 2019), respectively.

The current study's objective was to investigate the incidence of CLA in sheep in Ismailia Governorate and elucidate the virulence gene in the isolates and further examine the status of drug resistance genes in the isolated *C. pseudotuberculosis*. Secondly, it was to determine hematological and serum biochemical features in sheep infected by *C. pseudotuberculosis*.

MATERIALS AND METHODS

Sampling

Pus samples were collected from (100) sheep abscesses in superficial lymph nodes. The outer surfaces of the affected lymph nodes were cleaned, disinfected with 70% ethanol, and incised using a sterile *bla*de. The abscesses were squeezed, and paired swabs were collected from each lesion; the first was for direct microscopy, while, the second was for isolation and identification of the causative agents. Samples were subjected to bacteriological examination according to the method described before (Quinn *et al*., 2011).

Blood samples from sheep with the positive result of *Corynebacterium* spp. under a microscope were taken from the jugular vein; one sample was taken with anticoagulant for hematological examination and the other for serum separation and biochemical analysis

Bacteriological studies

Isolation and identification

The swabs which positive for *Corynebacterium* under the microscope were streaked in duplicate for each sample onto plates of brain heart infusion (BHI) agar (Oxoid) and 5% sheep blood agar (Oxoid) and both plates supplemented with fosfomycin (200 mg/litter) and nalidixic acid (4 mg/L), Nutrient agar; MacConkey agar (MAC), Mannitol salt agar (MSA) as a selective media. According to previously described methods. The bacterial culture was incubated aerobically for 24 and 48 hours at 37˚C (Songer and Post, 2004). The isolates were screened microscopically using Gram stain for the characteristic arrangements in a "V" formation (forming "Chinese letters") as single or in pairs, to confirm a pure bacterial culture used for subsequent analysis, the plate was checked to contain only one single type of colony (yellowish white, opaque, hemolytic, and convex colonies). A Gram stain was prepared from the isolate and used to confirm the bacteria morphology, size, and purity of the growth. The isolates were also studied biochemically with API Coryne (BioMe´rieux, Marcy l'Etoile, France) as per kit instruction. Catalase test, urease test and nitrate reduction test.

Antimicrobial sensitivity patterns of *C. pseudotuberculosis* isolates were determined using the Kirby-Bauerdisk diffusion method (Quinn *et al*., 1994). The isolates were tested for susceptibility to 9 antibiotics (Oxoid) (enrofloxacin (5 µg), amoxicillin (10 µg), amoxicillin-clavulanic acid (20/10 µg), trimethoprim-sulfamethoxazole (25 µg), norfloxacin (10 µg), ciprofloxacin (5 µg), gentamicin (10 µg), florfenicol (30 µg) and clindamycin (2 µg)).

Molecular diagnosis of *C. pseudotuberculosis* was carried out in Biotechnology Department, Animal Health Research Institute, Dokki, Giza, Egypt as the following: Out of identified *C. pseudotuberculosis*, 7 selected isolates were grown on BHI agar (Oxoid) for 48 hours at 37°C followed by extraction of the bacterial DNA using QI Aamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56°C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer.DNA Using the extracted bacterial DNA, virulence gene Phospholipase D (*Pld*), *rpoB* gene, sigma factor E (sigE), and drug resistance genes such as β-lactamase resistance gene (*bla*), integron (int), and aminoglycosides resistance gene (*aadA2*) were amplified using the primers listed in the Table 1.

Amplification reactions were prepared in 25 µl containing PCR master-mix (2X premix) (Emerald GT master mix, Takara kit) 12.5 µl, forward and reverse primers 1 µl, template DNA 6 µl completed to 25 µl by 4.5 µl PCR grade water. PCR was performed in a T3 Thermal cycler (Biometra), using *C. pseudotuberculosis* as the control positive (reference strain), while, sterile DNAse/RNAse free and Diethyl Pyrocarbonate (DEPC) water was used instead

of template DNA as the control negative. The reaction condition was initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94 °C for 30 sec., annealing (at 56°C for 30 sec. for *Pld*; 55°C for 40 sec., for *rpoB* and int; 58°C for 40 sec., for 16S rRNA, and at 50°C for 40 sec. for *bla* and *aadA2*) and extension at 72°C for 30 sec and 45 sec. followed by a final extension at 72°C for 10 min. The PCR was performed using DNase/RNase-free DEPC water instead of the template while positive control was performed using a DNA template from reference strain *C. pseudotuberculosis*. The amplified PCR products were electrophoresed through 1.5 Agarose gel in TBE buffer in Gel Casting Apparatus (Biometra) for 1 hour against 100 bp DNA ladder (Gel Pilot 100 bp ladder, Cat. no. 239035) supplied from QIAGEN (USA). The DNA bands were stained with Ethidium bromide and visualized using a gel documentation system, and the data were analyzed using computer software.

Clinicopathological studies

Hematological analysis

Two ml of blood samples were collected into EDTA tubes for the investigation of hematological parameters. The total RBC count was manually done by using a Neubauer-ruled hemocytometer. Hemoglobin (Hb) concentration was determined using the method of cyanomethemoglobin (J.T. Baker, London, England). Packed cell volume (PCV) was determined by microhematocrit centrifugation. The value of PCV was determined by using a built-in reader. The erythrocytic indices including mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC), were calculated from the value of total RBCs count, Hb concentration, and PCV by using the standard formulas (Lewis *et al*., 2006).

Biochemical analysis

Five ml of blood was collected into plain test tubes without anticoagulant. It was used for the separation of serum samples for biochemical investigations. Blood was left at room temperature for 2 h to be clotted and then centrifuged at 3000 rpm for 10 min. The collected sera were assayed for serum biochemistry. The level of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) (Reitman and Frankel, 1957), alkaline phosphatase (ALP) (Tietz *et al*., 1983), creatinine (Henry, 1979), urea (Patton and Crouch, 1977), calcium (Ferguson *et al*., 1962), potassium (Henry, 1974) and Inorganic phosphorus (El-Merzabani *et al*., 1977) were spectrophotometrically assayed using a semi-automated spectrophotometer (Erba-Chem7, Germany)and commercial kits (Spectrum, Cairo, Egypt).

Protein electrophoresis was done using SDS-Polyacrylamide gel electrophoresis according to Laemmli, (1970) in the animal health research institute Biochemistry department.

Statistical Analysis

The obtained data were statistically analyzed by an ANOVA (one-way) variance method considering $P < 0.05$ using MiniTab17© software. The significant differences were taken to Fisher multiple range tests to compare the means (Ryan and Joiner, 2005).

RESULTS

Caseous lymphadenitis in sheep

Isolated *C. pseudotuberculosis* was 14 out of 100 sheep with abscesses reaching 14%.

Distribution of C. pseudotuberculosis in sheep´s lymph nodes

Out of 14 affected sheep, the distribution of *C. pseudotuberculosis* in lymph nodes was Parotid 6/14 (42.9%), Mandibular 5/14(35.7 %), Prescapular 2/14 (14.3 %) and Prefemoral lymph node 1/14 (7.1%).

Bacteriological characters of C. pseudotuberculosis isolates

All isolates of *C. pseudotuberculosis* recovered from lymph nodes abscesses were Gram-positive coccobacilli to short bacilli, non-sporulated, non-capsulated, non-motile and appeared single, pairs in acute angles (like Chinese letter) and palisades arrangements. After 48 hours of incubation on blood agar, the colonies were white, smooth, opaque, circular, small in size, dry, waxy in appearance, and surrounded by a narrow zone of beta hemolysis. The colonies on BHIA, (after 48 hours) had the same character but were larger. The colonies were easily pushed across the agar surface and spattered when placed in flame.

Table 1. Primers sequences, target genes, amplicon sizes and cycling conditions.

Target agent	Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
					Secondary denaturation	Annealing	Extension		
Corynebacterium pseudotuberculosis	*Pld*	ATA AGC GTA AGC AGG GAG CA ATC AGC GGT GAT TGT CTT CCA GG	203	94˚C 5 min.	94˚C 30 sec.	56˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	Ilhan *et al*. (2013)
	rpoB	CGWATGAACATYGGBCAGGT TCCATYTCRCCRAARCGCTG	406	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 40 sec.	72˚C 10 min.	Sammra *et al.* (2014)
	sigE	GGMACCGCAGCDTTCGACGC CGTCCRCGGTGRATWCGGGA	490	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	Pacheco *et al*. (2012)
	bla	ATGAAAGAAGTTCAAAAATATTTAGAG TTAGTGCCAATTGTTCATGATGG	780	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	Catalán *et al*. (2010)
	AadA2	TGTTGGTTACTGTGGCCGTA GATCTCGCCTTTCACAAAGC	622	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	Walker *et al*. (2001)
	16S rRNA	ACCGCACTTTAGTGTGTGTG TCTCTACGCCGATCTTGTAT	816	94˚C 5 min.	94˚C 30 sec.	58˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	Pacheco *et al*. (2007)
	Integron	TGCGGGTYAARGATBTKGATTT CARCACATGCGTRTARAT	491	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	White *et al*. (2000)

Biochemical identification

All the isolates were positive for urease, and catalase while negative for nitrate reduction, and gelatin liquefaction while the results of sugar fermentation tests were glucose positive, trehalose negative and other sugars were variable results.

Antimicrobial susceptibility patterns of C. pseudotuberculosis recovered from abscess

A total of 14 *C. pseudotuberculosis* isolates were tested for susceptibility to 9 antibiotics. As shown in Table 2, the susceptibility pattern of *C. pseudotuberculosis* to antimicrobial agents varied among isolates according to (CLSI, 2015). The majority of isolates were highly sensitive to ciprofloxacin 13(92.86%) followed by florfenicol, trimethoprim/ sulfamethoxazole with a percentage of (85.71%). while such strains showed high resistance to amoxicillin/clavulanic acid and Clindamycin at a percentage of (71.42%).

Molecular characterization of C. pseudotuberculosis

Detection of *C. pseudotuberculosis* 16S rRNA

Out of the 14 isolates, 7 isolates were selected for investigation of the presence of 16S rRNA. All selected isolates of *C. pseudotuberculosis* gave positive results for the amplification of 16S rRNA of 816 bp fragment using oligonucleotide primers specific for *C. pseudotuberculosis* (Fig. 1).

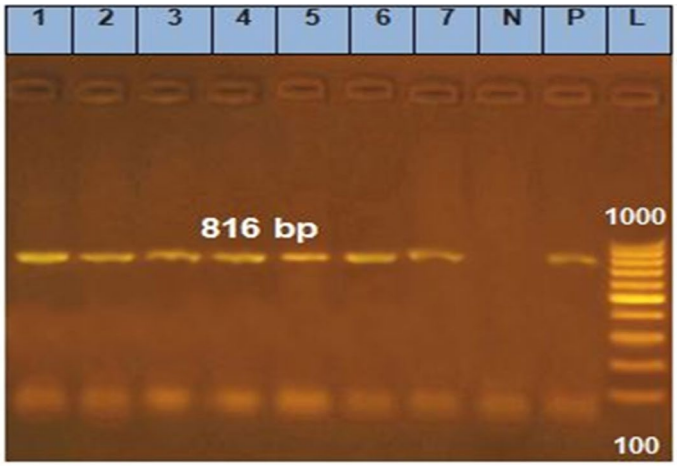

Fig. 1. Agarose gel electrophoresis showing amplification product of 16S rRNA of 816 bp (positive in all isolates) of *C. pseudotuberculosis* amplified using specific primers. Lane (L) indicates the DNA 100 bp ladder, (P) is a positive control, and (N) is negative control.

Detection of *C. pseudotuberculosis* virulence (*rpoB*) genes

All selected isolates of *C. pseudotuberculosis* gave positive results for the amplification of the *rpoB* gene of 406 bp fragment using oligonucleotides primers specific for *C. pseudotuberculosis rpoB* in 100% of tested isolates (Fig. 2).

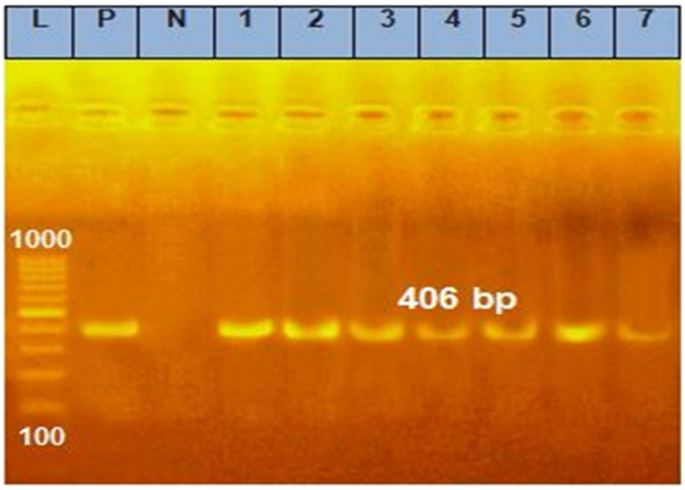

Fig. 2. Agarose gel electrophoresis showing amplification product of *rpoB* gene of 406 bp (positive in all isolates) of *C. pseudotuberculosis* amplified using specific primers. Lane (L) indicates the DNA 100 bp ladder, (P) is a positive control, and (N) is a negative control.

Detection of *C. pseudotuberculosis* virulence gene (*Pld*)

Six of the selected isolates of *C. pseudotuberculosis* gave positive results for the amplification of *Pld* gene of 203 bp fragment using oligonucleotide primers specific for *C. pseudotuberculosis Pld* in 85% of tested isolates (Fig. 3).

Detection of *C. pseudotuberculosis* of sigma factor E (sigE) gene

Amplification of sigE gene 490bp was detected in two isolates (no, 1 and 2) (Fig. 4).

Detection of beta-lactam resistance gene (*bla*) in *C. pseudotuberculosis*

All the selected isolates were positive for the amplification of the beta-lactam resistance gene (*bla*) at 780 bp fragment (Fig. 5).

Table 2. Results of Antimicrobial sensitivity test of *C. pseudotuberculosis* isolates (n=14) recovered from sheep external abscess according to the CLSI (2015).

Antimicrobials	Susceptible isolates		Resistance isolates	
	No.	%	No.	%
Ciprofloxacin	13	92.86	1	7.14
Florfenicol	12	85.71	2	14.28
Trimethoprim/sulfamethoxazole	12	85.71	2	14.28
Norfloxacin	6	42.86	8	57.14
Clindamycin	4	28.57	10	71.42
Gentamycin	8	57.14	6	42.86
Enrofloxacin	6	42.86	8	57.14
Amoxicillin/Clavulanic acid	4	28.57	10	71.42
Amoxicillin	6	42.86	8	57.14

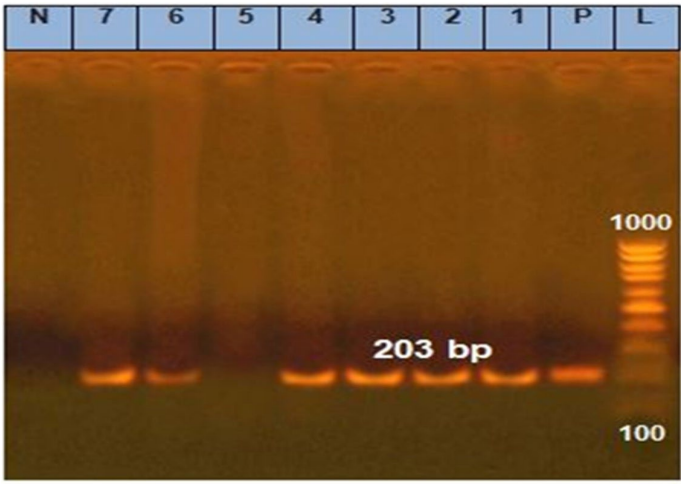

Fig. 3. Agarose gel electrophoresis showing amplification product of *C. pseudotuberculosis Pld* gene performed with specific primers. Lane (L) indicates the DNA 100 bp ladder, (P) is a positive control, and (N) is a negative control, lanes 1 to 7 are isolates from sheep. Amplicons of the *Pld* gene were detected in 1, 2, 3, 4, 6, and 7 tested isolates at 203 bp.

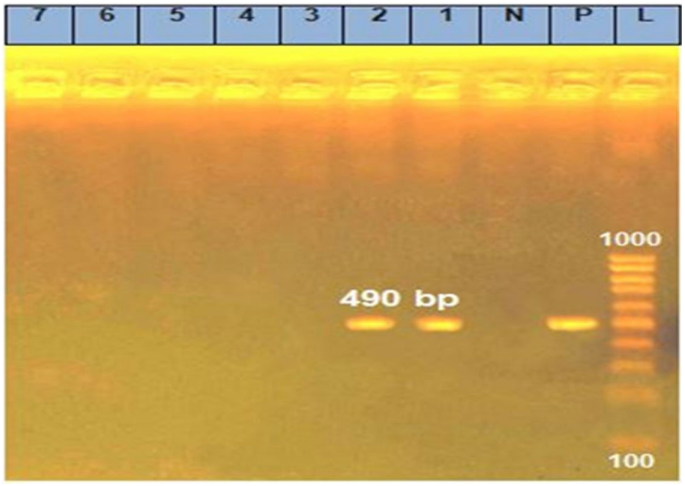

Fig. 4. Agarose gel electrophoresis showing amplification product of *C. pseudotuberculosis* sigE gene performed with specific primers. Lane (L) indicates the DNA 100 bp ladder, (P) is a positive control, and (N) is a negative control, lanes 1 to 7 are isolates from sheep. Amplicons of the sigE gene were detected in 1, and 2 tested isolates at 490bp.

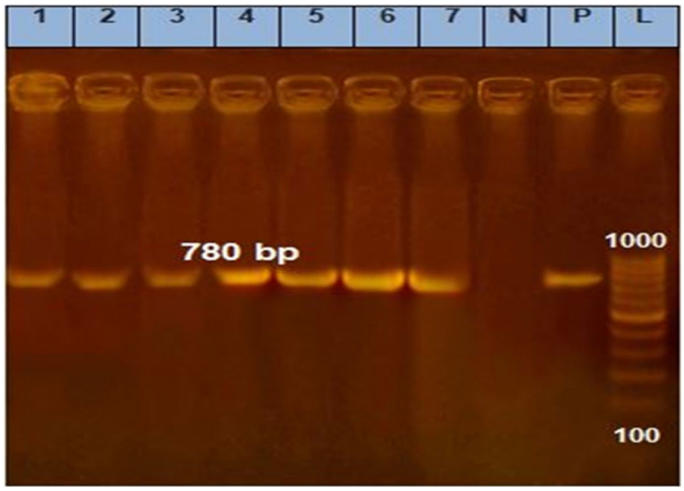

Fig. 5. Agarose gel electrophoresis showing amplification product of 780 bp, of beta-lactamase (*bla*) resistance gene of *C. pseudotuberculosis* performed with specific primers Lane (L) indicates the DNA 100 bp ladder, (P) is a positive control, and (N) is a negative control, lanes 1to 7 are isolates from sheep. Amplicon (*bla*) gene was detected in all lanes.

Detection of *C. pseudotuberculosis aadA2* genes

Amplification of the *aadA2* gene 622 bp was detected in four isolates (no. 1, 2, 3, and 5) (Fig. 6).

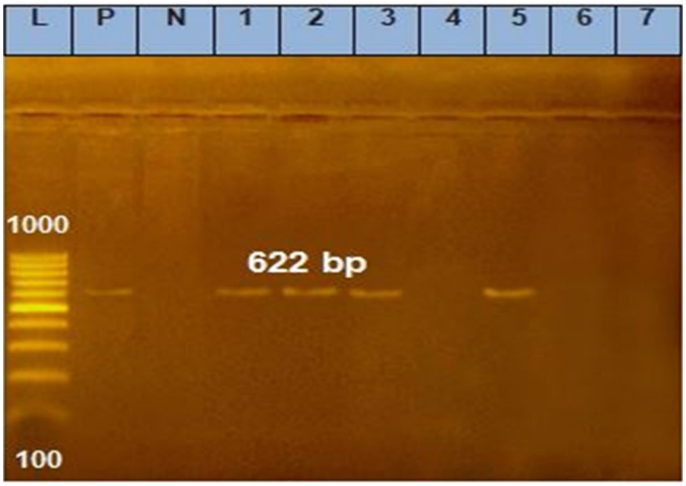

Fig. 6. Agarose gel electrophoresis showing amplification product of aminoglycoside (*aadA2*) resistance gene 622 bp (positive in lanes 1, 2, 3, and 5) of *C. pseudotuberculosis* amplified using specific primers. Lane (L) indicates the DNA 100 bp ladder, (p) is a positive control, and (N) is a negative control.

Detection of *C. pseudotuberculosis* of integron (int) genes

Amplification of int gene 491bp was detected in all isolates (Fig. 7)

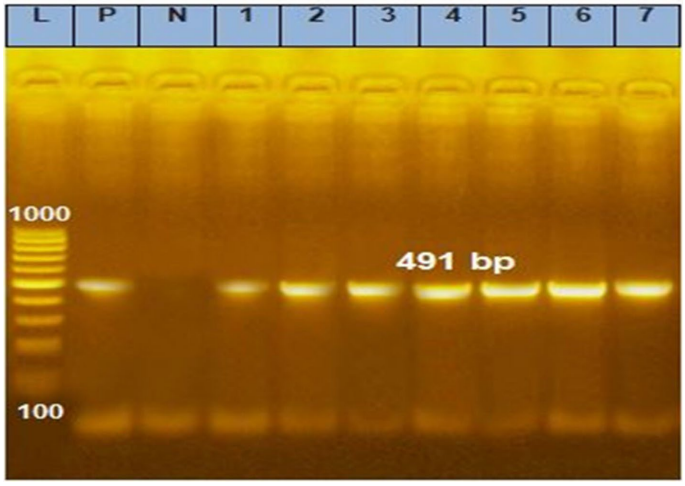

Fig. 7. Agarose gel electrophoresis showing amplification product of integron 491 bp (positive in all isolates) of *C. pseudotuberculosis* amplified using specific primers. Lane (L) indicates the DNA 100 bp ladder, (P) is a positive control, and (N) is a negative control.

Hematological findings

hematological findings in Table (3) revealed a significant decrease in RBCs count, Hb conc., and MCHC in *C. pseudotuberculosis*-infected group as compared with apparently healthy sheep. Also, there was leucocytosis and lymphocytosis. neutrophilia, eosinophilia, monocytosis, and basophilia in *C. pseudotuberculosis*-infected group as compared with apparently healthy sheep.

Biochemical results

Table 4 showed in the group naturally infected with *C. pseudotuberculosis* a significant increase in the level of AST, ALT, ALP, Cholesterol, LDH, urea, Phosphorus concentration, and Creatinine as compared to the healthy sheep. However, infection with *C. pseudotuberculosis* showed a significant decrease ($p<0.05$) in total protein, albumin, globulin, Glucose, and calcium compared

with apparently healthy sheep. However, there were no significant changes in potassium (K^+).

DISCUSSION

Caseous lymphadenitis disease of sheep manifests itself in two forms the external and the internal forms. The most frequent of which is the external form of CLA, characterized by abscess formation in superficial lymph nodes and subcutaneous tissues (Baird and Fontaine, 2007). The external form of this disease was resulting in decreasing general body performance, low production of wool, and carcass condemnation (Arsenault *et al*., 2003). *C. pseudotuberculosis* causes abscesses of external and internal lymph nodes and inner organs of sheep and goats as well as those of Old World and New World camelids (Wernery and Kinne, 2016; Torky *et al*., 2023). The final stage of the disease is characterized by general wasting and emaciation of the infected animals (Braga, *et al*., 2006; Sting *et al*., 2017). Caseous lymphadenitis (CLA) is a chronic disease caused by the bacterium *Corynebacterium pseudotuberculosis* that affects small ruminants worldwide (Burmayan and Brundage, 2021). CLA is widely distributed which causes farmers to suffer significant financial losses due to loss of fertility, decreased milk and meat production, hide and wool loss, culling of infected animals, and carcass condemnation (Costa *et al*., 2022). CLA affects industries worldwide and proves to be a hindrance to animal health and disease prevention (Burmayan and Brundage, 2021). The occurrence of the disease is dependent on immunity, which can be altered by gestation, lactation, immunosuppressive disease, nutrition, and other management factors to which both sexes are subject (Oreiby *et al*., 2014; Burmayan and Brundage, 2021). *C. pseudotuberculosis* can survive in the environment for up to 6 months, and CLA is a long-term disease beyond the infected animals that remains in this condition for the duration of their lives, disseminating the agents through purulent discharge of LNs (O'Reilly *et al*., 2008).

In the current study, the investigation of the prevalence of the external form of CLA resulted in an overall prevalence of 14% in sheep. These results are consistent with a previous study that reported a prevalence of CLA in sheep and goats were 13.5% (Menzies and Muckle 1989; Abd El Tawab *et al*., 2019). The prevalence of *C. pseudotuberculosis* in this study is somewhat higher than the reported results in other localities in Egypt as a prevalence of 6.7% and 10%were reported in El-Gharbia governorate

Table 3. Hematological parameters in apparently healthy and *C. pseudotuberculosis* infected sheep.

Parameters \ Groups	apparently healthy (n.=10)	*C. pseudotuberculosis* (n.=14)
RBCs ($\times10^6$/µl)	12.27±0.44[a]	10.20±0. 3[b]
PCV (%)	20.33±0.05[a]	34.26±0.06[b]
Hb concentration (g/dl)	6.83±0.88[a]	4.01±0.43[b]
MCV (fl)	165.7±0.44[b]	335.9±2.4[a]
MCH (pg)	55.66±2.45[a]	39.31±3.14[b]
MCHC (%)	33.59±5.7[a]	11.07±3.77[b]
WBC ($\times10^3$/µl)	5.89±0.72[b]	8.19±2.32[a]
Neutrophil ($\times10^3$/µl)	3.78±0.39[b]	5.17±1.33[a]
Lymphocyte ($\times10^3$/µl)	1.63±0.21[b]	2.98±0.28[a]
Monocyte ($\times10^3$/µl)	0.35±0.05[b]	0.70±0.09[a]
Eosinophil ($\times10^3$/µl)	0.20±0.08[b]	0.28±0.11[a]
Basophil ($\times10^3$/µl)	0.06±0.01[b]	0.08±0.02[a]

Means within the same row with different superscripts are significantly different (P<0.05

Table 4. Biochemical parameters in apparently healthy and C. pseudotuberculosis infected sheep.

Parameters \ Groups	Apparently healthy (n=10)	*C. pseudotuberculosis* (n=14)
Total protein (g/dl)	7.59±0.3[a]	4.56±0.48[b]
Albumin (g/dl)	3.62±0.28[a]	2.25±0.31[b]
Globulin (g/dl)	3.97±0.38[a]	2.31±0.37[b]
α– globulin (g/dl)	1.32±0.58[a]	1.11±0.45[b]
β– globulin (g/dl)	0.85±1.04[a]	0.60±1.00[b]
λ– globulin (g/dl)	1.80±0.95[a]	1.20±0.85[b]
Glucose (mg/dl)	45.0±2.5[a]	35.0±2.0[b]
Cholesterol (mg/dl)	157.0±11.2[b]	169±21.2[a]
LDH (U/l)	720.45±30.6[b]	962.20±158.24[a]
ALT (U/l)	10.0±0.4[b]	33.0±0.62[a]
AST (U/l)	124.0±2.5[b]	175.0±2.6[a]
ALP (U/l)	42.0±0.45[b]	76.0±0.67[b]
Urea (mg/dl)	22.06±0.63[b]	30.33±0.76[a]
Creatinine (mg/dl)	0.57±0.068[b]	0.60±0.067[a]
Calcium (mmol/l)	1.98±0.03a	1.24±0.51[b]
P (mg/dl)	3.66±0.2[b]	5.0±0.27[a]
K+ (mmol/l)	3.78±0.46[a]	3.82±0.53[a]

Means within the same row with different superscripts are significantly different (P<0.05

(Oreiby *et al*., 2014) and Matrouh Governorate (Torky *et al*., 2023) and lowers than the results in other localities in Egypt as a prevalence of 41.3% in Menoufia Governorate (Algammal, 2016). These variations in prevalence between the current study region and other regions in Egypt may be attributed to the management system and climatic conditions in each region including the ambient temperature which affects the viability and spread of the bacteria (Al-Gaabary *et al*., 2010).

Furthermore, the prevalence of the disease in sheep was 14% which may be attributed to the small cuts in the skin that occurred during sheep shearing let sheep more prone to *C. pseudotuberculosis* infection. This trend of prevalence in small ruminants in the anterior part of the body is in agreement with previous results (Menzies and Muckle 1989; Pandey *et al*., 2007; Abd El Tawab *et al*., 2019). In sheep the predilection site of *C. pseudotuberculosis* localization is mainly in the head region as out of 14 affected sheep, the distribution of *C. pseudotuberculosis* in lymph nodes was Parotid 6/14 (42.9%), Mandibular 5/14 (35.7%), Prescapular 2/14 (14.3 %) and Prefemoral lymph node 1/14 (7.1%). These results in parallel with (Oreiby *et al*., 2014) revealed that parotid lymph node lesions in sheep were the most commonly affected lymph node where it was found affected in 52.17%of lesions followed by mandibular lymph nodes (22.60%), superficial cervical subcutaneous lesion (4.34%), retropharyngeal lymph node (2.60%) and finally the least affected site was popliteal lymph node (0.86%). On the other hand, (Abd El Tawab *et al*., 2019) reported that the distribution of *C. pseudotuberculosis* localization in lymph nodes was Mandibular 10 (43.5%), Parotid 9 (39.1%), Prescapular 3 (13%), and Prefemoral 1 (4.3%).

CLA may become endemic within herds or flocks due to its resistance to certain antibiotics, such as ampicillin, clindamycin, and doxycycline HCl (Abebe and Tessema, 2015); its constantly increasing ability to survive in harsh environments for extended periods; and the limitations associated with detecting subclinically infected animals (Williamson, 2001). In most cases, treatment of the external forms of CLA involves the identification of infected animals before abscess rupturing to prevent contamination of the environment and soil. Once identified, isolation of the affected animals is required to avoid the further spread of the disease. These methods were consistent with our study and have shown to be successful in preventing the further spread of infection. Regarding antimicrobial susceptibility of *C. pseudotuberculosis* revealed highly sensitive to ciprofloxacin 13 (92.86%) followed by florfenicol, trimethoprim/ sulfamethoxazole with a percentage of 85.71% is agreement with (Abdulrahman, 2021). While such strains showed high resistance to amoxicillin/clavulanic acid and Clindamycin at a percentage of 71.42%. Indeed, in Argentina, quinolones have been reported to be effective on 100 % of *C. pseudotuberculosis* isolates (Algammal, 2016; Gallardo *et al*., 2019). This difference in the microorganism susceptibility to Quinolones may be attributed to the differences in antibiotics regimes used in the treatment of bacterial infection or even the use of antibiotics as growth promoters in intensive production in livestock or even in broilers that led to the emergence of Quinolones resistance genes in the environment that by somehow find their way to be transformed to pathogenic bacteria including *C. pseudotuberculosis*. On the other hand, the isolates have high resistance to beta-lactam amoxicillin-clavulanic (71.42%). followed by Amoxicillin at 57.14% in addition to Aminoglycoside, and Gentamycin at 42.86% following the observed low activity of β-lactam and aminoglycoside (Gallardo *et al*., 2019). Surprisingly, the isolate's resistance to the Florfenicol is only about 14.28%. The high susceptibility of *C. pseudotuberculosis* isolates to phenicol in this study is by the increased sensitivity pattern of bacteria to chloramphenicol. This is in line with the assumption drown by (Tatavarthy *et al*., 2012; Algammal, 2016) that antibiotic recycling by reusing traditional drugs (e.g., tetracycline, chloramphenicol, and trimethoprim) should be carefully considered. This may indicate changing trends in the antibiograms of all bacteria especially *C. pseudotuberculosis* isolates in Egypt. This probably demands reconsideration for the use of chloramphenicol in *C. pseudotuberculosis* infection treatment instead of ciprofloxacin or third and fourth-generation Cephalosporins in light of the increasing resistance to quinolones and β-lactam. Moreover, continuous monitoring of resistance patterns is essential for the successful treatment of *C. pseudotuberculosis* infection between animals or humans.

The molecular identification with primers targeting the 16S rRNA genes of *C. pseudotuberculosis* yielded rapid and precise results (Torky *et al*., 2023). In the current study, PCR was used for the detection and amplification of the 16S rRNA gene to confirm the diagnosis of the isolated strains. The 16S rRNA gene is the gene of choice for most microbial taxonomy studies; therefore, this gene is useful for estimating the prevalence of *C. pseudotuberculosis* in the animals studied (Çetinkaya *et al*., 2002; Abdulrahman *et al*.,2020). All the selected *C. pseudotuberculosis* isolates were positive for the 16S rRNA gene with the specific 816 bp amplicon size. In addition, amplification of the gene in the obtained isolates was also carried out. All the examined isolates were positive with the specific amplicon size (406 bp). These results agreed with those reported by other studies (Pacheco *et al*., 2007; Pavan *et al*., 2012; Algammal, 2016). PCR detection of both 16S rDNA and *rpoB* genes helps in the differential diagnosis of *C. pseudotuberculosis* from other pyogenic pathogens that might present in pus discharge (Pacheco *et al*., 2007; Abdulrahman *et al*., 2020). Another study carried out the identification of 57 isolates of *C. pseudotuberculosis* by bacteriological tests and the amplification of 16S rRNA, *rpoB*, and *Pld* genes, as well as, genes involved in virulence and pathogenicity (Guerrero *et al*., 2018). The virulence factor *Pld* and cp40 continue to be an objective of study due to their potential for diagnostic and vaccine development (Rodríguez-Domínguez *et al*., 2022). Searching for the virulence genes of *C. pseudotuberculosis*, found that six tested isolates out of seven isolates express the virulence gene Phospholipase D (*Pld*). This gene is responsible for the production of the protein phospholipase which is an exotoxin that dissociates the cell membrane sphingomyelin and hence increases the vascular permeability leading to the spread of *C. pseudotuberculosis* and its invasion to the tissue and hence its transportation through the macrophage to the regional lymph nodes (Baird and Fontaine 2007, Corrêa *et al*., 2018; Abd El Tawab *et al*. 2019). Thus, *Pld* is considered the most important virulence factor in the pathogenesis of *C. pseudotuberculosis*-produced diseases (Hodgson *et al*., 1999).

Concerning the antibiotic-resistance genes the investigation of the expression of these genes in the selected isolates revealed the absence of the integron from all isolates. This result is in agreement with the reported results of negative results for integron amplification in 100% of *C. pseudotuberculosis* isolates (Gallardo *et al*., 2019). Searching for the β-lactam resistance gene (*bla*) revealed the absence of the gene in all isolates (100%) which is consistent with the results of the antibiotic susceptibility test that the bacteria under study are resistant to the beta-lactam members. However, the current study results indicate the resistance of *C. pseudotuberculosis* to this drug family in parallel with a study showing the microorganism's low susceptibility to β-lactam (Gallardo *et al*., 2019). These results may suggest the acquisition of the resistance gene due to continuous exposure of the microorganism to β-lactam. The aminoglycosides-resistant gene amplification in the selected isolates revealed the expression of the *aadA2* gene in four out of seven isolates which is consistent with low susceptibility of *C. pseudotuberculosis*. In the current study, 42.86% of the isolates proved to be resistant to gentamycin. This result also is in parallel with the presented data (Gallardo *et al*., 2019). Indeed, gentamicin was shown to be effective on the majority of *C. pseudotuberculosis* isolates (Connor *et al*., 2000).

Laboratory analysis of bodily fluids such as blood via different means, hematology, and blood biochemistry is of great value to validate predictive disease diagnosis and/or prognosis; this applies to infectious diseases. CBC is the most commonly performed blood test for clinical or research purposes that provide an overview of a patient's general health status and can also indicate the presence of any kind of disease. In any research, monitoring

blood biochemical parameters is crucial to detect any changes in an early period and it can be of greater help to anticipate preliminary results, even though there are no obvious symptoms that manifest on clinical inspection.

Hematological findings of apparently healthy and *C. pseudotuberculosis* bacteria infected groups were reported in Table (3), with a significant decrease in RBCs count, Hb concentration, and MCHC percentage in *C. pseudotuberculosis* bacteria infected groups that indicated the anemia was correlated with the severity of bacterial infection. These results were by the Previous studies (Jain, 2000; Junior *et al*., 2006; Ibtisam, 2008; Osman *et al*., 2012; Othman *et al*., 2014; Mahmood *et al*., 2015; Odhah *et al*., 2017) reported similar findings to our study where there were significant changes in Hb concentration, RBC count, MCV, and MCHC concentrations. These results could be inferred as harmful effects of *C. pseudotuberculosis* exotoxin, on endothelial cells of the vascular system disrupting the normal physiology of the hemopoietic system. This result is in agreement with (Russell and Grindem, 2000) reported severe hemolytic anemia, macrocytic hypochromic, and hypochromic normocytic anemia observed in sheep experimentally infected with *C. pseudotuberculosis*.

Naturally, CLA-infected sheep showed a significant increase in WBC count due to the increased neutrophil, monocyte, and lymphocyte count (Junior *et al*., 2006; Ibtisam, 2008; Mahmood *et al*., 2015; Odhah *et al*., 2017). Similarly, in this study WBC, neutrophil, monocyte, and lymphocyte counts were significantly high with the *C. pseudotuberculosis* infection. These findings are in accord with previous studies' findings (Ibtisam, 2008; Osman *et al*., 2012; Othman *et al*., 2014; Mahmood *et al*., 2015; Odhah *et al*., 2017) who stated that *C. pseudotuberculosis* resulted in a significant increase in the parameters under the study in sheep of CLA. The production of proinflammatory cytokines in response to the *C. pseudotuberculosis* antigen leads to chemotaxis of other inflammatory cells mainly macrophages and lymphocytes to the site of inflammation. IL-1β has many roles which consider a leukocytic pyrogen that induces T activation and B cell differentiation, which is a crucial step in developing a cellular immune response against this pathogen. Additionally, IL-1β induces the release of other cytokines such as TNF and IL-2. In the current study, eosinophil and basophil counts were significantly increased in groups. This study hypothesized that the significant increase in the basophil count is due to the cellular immunity response. Hence, *C. pseudotuberculosis* is a facultative intracellular pathogen and can live inside the macrophages. These results were similar to previous reports (Othman *et al*., 2014; Mahmood *et al*., 2015; Odhah *et al*., 2017). In contrast, a result that disagreed with Ibtisam (2008) and Osman *et al*. (2012) both studies have reported no significant changes in the basophils count during CLA in sheep.

Blood biochemistry assessment coordinated with the hemogram and the leukogram revealed various significant changes post-infection with *C. pseudotuberculosis* in the current investigation.

ALT and AST are the most elevated enzymes in liver diseases. Both enzymes could be elevated before any clinical disease is apparent such as in acute hepatic necrosis and their levels may reach as high as 100 times the normal level with peak activity between 7 and 12 days (Burtis *et al*., 2008).

In the study, liver enzymes such as ATL, AST, and ALP were significantly changed in *C. pseudotuberculosis*-infected group. These findings agreed with those described by some authors (Burtis *et al*., 2008; Ibtisam 2008; Hall, and Cash, 2012; Osman *et al*., 2012; Othman *et al*., 2014; Mahmood *et al*., 2015; Odhah *et al*., 2017) stated that bacterial invasion-induced liver damage, hepatotoxicity, obstruction of the biliary tree, disease processes that involved hepatocytes integrity and hemolytic diseases can significantly change ATL, ALP, AST, and GGT serum concentrations. We hypothesize that the high fluctuation in liver enzymes can reflect the severity and/or the chronic nature of the disease affecting the liver. Hence, *C. pseudotuberculosis* inoculation led to abscess formation in the liver.

Urea is the end product of the protein catabolism process in the body and it is cleared by the kidneys. Measurement of plasma urea concentration reflects the kidney function. Additionally, creatinine is the end product of creatine phosphate breakdown in the muscles. Both creatinine and urea are cleared from the body using the kidneys through the process of glomerular filtration (Burtis *et al*., 2008). In our study, creatinine, and urea concentrations were significantly changed in the *C. pseudotuberculosis* naturally infected sheep. This result is parallel with (Ibtisam, 2008) who reported the significantly high level of serum Creatinine and blood urea concentrations in sheep naturally infected with CLA. This could be attributed to the degenerative effect of *C. pseudotuberculosis* toxin leading to impairment of renal function. This result is in agreement with Osman *et al*. (2012) and Othman *et al*., (2014) who reported that the increase in these parameters may be due to the infection of *C. pseudotuberculosis* which may lead to muscle damage due to the formation of abscesses and also its effect towards the renal system. On the other hand, Mahmood *et al*. (2015) suggested that decreased blood flow into the kidneys could increase serum creatinine and urea concentrations.

Serum LDH is commonly found in the liver, skeletal muscles, heart, and kidneys. Hence, its level pattern changes due to tissue damage and it can be considered a marker of the instability of cellular integrity or cell death caused by a pathological condition (Al-Saadoon *et al*.,2003; Othman *et al*., 2014; Mahmood *et al*., 2015). In this study, LDH was significantly changed with the *C. pseudotuberculosis* natural infection. It suggested that LDH is a precise indicator that has a positive association with liver diseases, heart problems, uric acid elevation, and hematocrit, and it is proposed to be a marker for cardiac dysfunction (Sreenivasan *et al*., 2010). Elevation in LDH serum concentration may raise from tissue damage by the toxic materials or pathological lesions in the liver, lymph nodes, lung, bone marrow, and spleen, and it's hypothesized that the LDH source in such cases may be the inflammatory cells (Al-Saadoon *et al*., 2003). Since, in this study, the pathophysiological mechanism of *C. pseudotuberculosis* infection involved inflammation, tissue damage, high blood urea, high PCV, and toxicity which were reflected in high serum LDH concentration. Moreover, the estimation of serum LDH level is a useful tool for the detection of acute myocardial infarction (Huijgen *et al*.,1997). Therefore, we believe that LDH can be used in the CLA diagnosis scheme; since, it's a very sensitive biomarker for tissue damage, especially in the lung, liver, and lymph nodes, major organs that are affected by CLA in small ruminants.

Calcium is the most common element in the body, and 99% of it is found in the skeleton. Calcium disorder is presented two main metabolic forms, hypocalcemia, and hypercalcemia. It is within the realm of science that hypocalcemia is due to a fall in free calcium, albumin-bound calcium, or both whilst hypercalcemia is due to the influx of the calcium from the calcium pool into the extracellular fluids more than its efflux (Burtis *et al*., 2008). In the current study, calcium concentration showed significant changes in infected sheep with *C. pseudotuberculosis*. This result was agreed with Othman *et al*., (2014) and Mahmood *et al*., (2015). These findings disagree with Osman *et al*. (2012) who stated there was no significant change in serum calcium concentration post-inoculation with *C. pseudotuberculosis*. This could be due to the toxin effect on the kidneys resulting in renal failure and leading to reduced glomerular filtration rate and retaining the electrolytes and some other substances in the blood. Calcium binds to albumin making albumin-bound calcium. Such chronic diseases that lead to lower serum albumin ensure low calcium levels by default contributing to hypocalcemia (Osman *et al*., 2012). Hence, the albumin concentration in this study was significantly decreased.

The best strategy for preventing the disease is immunization (Paton *et al*., 2003). It would be beneficial to further study currently available vaccines and their efficacies in sheep and goats (Dorella *et al*., 2009). This article shows the importance of monitoring and documentation during the time of an outbreak to better control the spread.

CONCLUSION

A right control measure is required to prevent the spread of abscessation among small ruminants as grazing among sharp objects and vigorous shearing to guard against producing entrance of the bacteria to the body. The current study revealed a change in the *C. pseudotuberculosis* susceptibility to the antimicrobial effect than the previous report These results may necessitate the reconsideration of the general *C. pseudotuberculosis* management protocol based on the current situation which seems to be also specific for each locality depending on the previous exposure to antibiotic regimes for controlling *C. pseudotuberculosis* or other microorganisms. *C. pseudotuberculosis* has resulted in quite significant changes in the CBC as observed in this study. This provides a better understanding of the blood profile following CLA and its important role in the disease occurrence.

CONFLICT OF INTEREST

The authors declare that they no conflict of interest.

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