RESEARCH ARTICLE

Prepared-Antigens based Serodiagnosis of Ovine Pseudotuberculosis in Serum and Milk of Lactating Ewes

Dalal Rizk[1], Atef Oreiby[1], Yamen Hegazy[1], Amin Tahoun[1], Hazim O Khalifa[2,3*], Magdy AL-Gaabary[1] and Tetsuya Matsumoto[4*]

[1]Department of Animal Medicine, Faculty of Veterinary Medicine, Kafrelsheikh University, Kafr El-Sheikh 33516, Egypt
[2]Department of Veterinary Medicine, College of Agriculture and Veterinary Medicine, United Arab Emirates University, Al Ain, P.O. Box 1555, United Arab Emirates
[3]Department of Pharmacology, Faculty of Veterinary Medicine, Kafrelsheikh University, Kafr El-Sheikh 33516, Egypt
[4]Department of Infectious Diseases, Graduate School of Medicine, International University of Health and Welfare, Narita 286-0048, Japan
*Corresponding author: hazimkhalifa@uaeu.ac.ae; tetsuya.m@iuhw.ac.jp

A B S T R A C T

Pseudotuberculosis is a non-treatable disease in sheep caused by *Corynebacterium pseudotuberculosis* resulting in significant economic losses. In this study, eleven sheep flocks underwent examination for signs of pseudotuberculosis, focusing on lymph nodes. Prevalence of the disease within the flocks ranged from 0 to 33.3%. Pseudotuberculosis abscesses were frequent in lymph nodes of the head and neck. Lesions were closed, varied in size, and contained pus with a consistency ranging from milky to greenish-creamy. Two different crude antigens of a PCR-identified local *Corynebacterium pseudotuberculosis* strain were prepared, tested as a separate ELISA solid phase to detect antibodies against *Corynebacterium pseudotuberculosis* in sera and milk of eight lactating ewes bred within pseudotuberculosis-endemic herds. Crude-exotoxin was filtered from cultured-broth supernatant and then concentrated. Sediment (the crude-somatic-antigen) was washed and sonicated. Both were quantified by the Bradford method. Comparative performance of both antigens to a commercial recombinant-phospholipase-D antigen was investigated through ELISA against reference positive and negative sera. A strong positive correlation between the antigens was evident. Antigens were used to detect antibodies in sera, whole milk, and cream-devoid milk. A positive correlation existed between results of serum and milk of the same animal. This study described the clinical nature of ovine pseudotuberculosis and showed the validity of the prepared antigens for serodiagnosis in serum as well as in milk which was not reported previously.

INTRODUCTION

Tenacious diseases such as pseudotuberculosis is a chronic bacterial threat for sheep, goats, and other warm-blooded animals such as camels and wild ruminants (Borham *et al.,* 2017; Di Donato *et al.,* 2024). Its etiological agent *Corynebacterium pseudotuberculosis* is a minor zoonosis and has unique characteristics being a Gram's-positive, of a long-survival period, mycolic acid-containing cell wall and facultative intracellular bacterium. It is also responsible for other disease conditions in different animal species (Oreiby *et al.,*

2014a). Like *Corynebacterium pseudotuberculosis*, other facultative intracellular zoonotic bacteria such as *Brucella spp.* are also reported in sheep (Hegazy *et al.*, 2022). Such types of bacteria are not treated in animals.

The disease has a serious negative economic impact on small ruminant production (Aftabuzzaman and Cho, 2021), as well as on the related industries. It results in carcass trimmings, reduced fertility, milk production and skin quality. In addition to 5% decrease in wool production during the first year after infection (Paton, 2010). Furthermore, the lowered price of diseased animals, cost of control, negative effects on international

trade, and being a minor zoonosis are other aspects to be considered in this respective.

Ovine pseudotuberculosis is a pyogenic disease-causing abscess therefore, it is occasionally termed abscess disease (Baazizi *et al.*, 2024). The lesions are usually found in lymph glands and infrequently in other sites. Abscess of ovine pseudotuberculosis may show alternative layers of pus surrounded by fibrous tissue capsules giving the characteristic onion-ring appearance of the lesion. The disease is endemic in Egypt with variable occurrence rates within and among different districts which is dependent on complex factors (Oreiby *et al.*, 2014b).

Affected sheep can spread the disease to many animals during the shearing season through the contamination of shearing cuts. The causative organism will enter through the damaged skin and mucous membranes to reach the associated regional lymph glands (Aftabuzzaman and Cho, 2021). Carrier and subclinical cases do exist and act as a hidden source of infection to the rest of the flock (Oreiby *et al.*, 2013). Moreover, *Corynebacterium pseudotuberculosis* can survive for up to six months in the environment and the diseased animals remain infected for almost their whole life span, disseminating the agent through purulent discharge of the affected lymph glands. Therefore, diagnosis of pseudotuberculosis must be the cornerstone to investigate the epidemiological situation and to build up a control plan, research investigations in this respect should be encouraged.

Lesions of the superficial lymph glands can be seen and sampled easily (Al-Gaabary *et al.*, 2009; Oreiby *et al.*, 2014b). However, abscesses may involve internal organs and their associated lymph glands. Abattoir surveys may be used to detect visceral pseudotuberculosis affections as well as similar lesions in different animal species after slaughter (Al-Gaabary *et al.*, 2010; Borham *et al.*, 2021). However, serological testing is tremendously important to predict these cases in alive animals, especially ELISA which is the most common serological test used for this purpose not only for sheep but also for other species such as camels (Borham *et al.*, 2016). Although radiography may be used to predict visceral lesions in living animals (Oreiby, 2015), but it is not suitable for screening purposes.

Various antigen preparations have been used in ELISA tests, including cell-surface and somatic antigens of *Corynebacterium pseudotuberculosis* (Paule *et al.*, 2003), total secreted antigens (Guimaraes *et al.*, 2009), recombinant proteins (Dos Santos *et al.*, 2022), or even other antigens (Oreiby, 2015). However, exotoxin is better suited than somatic antigen to detect both humoral and cellular responses against *Corynebacterium pseudotuberculosis* as it is a more sensitive solid phase ELISA antigen and produces higher IFN in blood cultures (Paule *et al.*, 2003; Meyer *et al.*, 2005).

Most of the previously published papers that contain serological investigations of *Corynebacterium pseudotuberculosis* infection were conducted on serum samples. Limited and very scarce studies were conducted on milk despite its validity in detecting humeral as well as cell-mediated responses against *Corynebacterium pseudotuberculosis*. A previous study

had shown the possible validity of detecting cellular response against this pathogen in milk through gamma-interferon assay in comparison to blood (Oreiby and Hegazy, 2016). However, based on our knowledge, limited studies, if any, investigated the comparative detection of humeral immune response in milk and serum simultaneously. Therefore, this study aimed at the production of two different *Corynebacterium pseudotuberculosis* antigens, and to compare their performance to detect humeral response in both milk and serum of lactating animals bred within endemic flocks, and to describe the disease in such flocks.

MATERIALS AND METHODS

Animals and *Corynebacterium pseudotuberculosis* seed strain: Eleven sheep mobile flocks in Qualin district, Kafrelsheikh governorate (Egypt) were examined. The superficial lymph glands of each animal were carefully palpated. Enlarged glands were aseptically aspirated by a wide needle and transported to the lab on ice. Bacteriological and serological confirmation of cases was performed (Rizk *et al.*, 2015). The used strain was identified by PCR targeting *pld* gene according to Pacheco *et al.* (2007).

Tested samples: Milk and plain blood samples were collected from eight lactating ewes. Animals in this region are usually bred for meat production, lactating ewes are scarce and limited only to a period of newborn suckling. Samples were sent to the lab on ice, each milk sample was divided into two parts; one was kept as whole milk, while the other part was centrifuged, and the milk cream layer was removed. Plain blood was centrifuged, and serum was separated. Milk and blood samples were kept at -20°C till they were used for serological investigation. Two reference positive and two reference negative serum samples were used in serological procedures, their status was confirmed by ELISA in a previous study (Oreiby *et al.*, 2013).

Exotoxin and somatic antigens preparation: The isolate was grown with shaking in 500ml of broth at 37°C for 48h. After centrifugation at 8000 rpm/10 minutes, the supernatant was filtered through a 0.22 μm filtration funnel and the exotoxin was concentrated by salting out using ammonium sulfate according to Tahoun *et al.* (2015). Somatic antigen was prepared according to Binns *et al.* (2007). Antigens were kept at -20°C after quantification according to the method described by Bradford (1976), using bovine serum albumin as a heterologous protein for this purpose.

Standardization of the prepared antigens: For standardization of the prepared antigens, it was compared with a standardized commercial rPLD antigen (Hyphen Biomed, France) against two reference positive and two reference negative sera. ELISA was utilized for this purpose. The test and its solutions were applied according to Menzies *et al.* (2004) with some modifications; each well was coated by 0.5μg prepared coating antigen in 50μl coating buffer. Two different coating solutions PBS (pH 7.4) and bicarbonate (pH 9.4) were tested to

determine the best one which resulted in better precipitation of the tested antigen according to its isoelectric point. Rabbit anti-sheep IgG (H+L)-HRP conjugate (Invitrogen Immunochemicals, USA) was used for the test. The plates were read at 415nm and 630nm as a reference wavelength (Dual reading mode). ELISA procedures on whole milk, cream-devoid milk, and serum samples were similar to that of reference sera.

Statistical analyses: The Pearson correlation among all tested antigens was calculated using SPSS version 19. Also, it was calculated for both coating buffers: carbonate bicarbonate buffer and PBS. All other statistical analysis was carried out using the Chi-square test and Fisher's test on SPSS V 19. Graphs were created by Microsoft Excel, and statistical significance was set as P<0.05.

RESULTS

Flock prevalence and clinical signs: The prevalence of ovine pseudotuberculosis varied among the investigated flocks, as outlined in Table 1. Affected animals exhibited abscesses in the external lymph glands of the head, neck, and thigh, as depicted in Fig. 1. These lesions were characterized by a closed appearance and contained white-greenish pus, occasionally appearing milky, but more commonly demonstrating a caseated consistency.

Table 1: Prevalence of ovine pseudotuberculosis among the eleven examined sheep flocks.

Flock No.	Total animals	Affected animals	Prevalence (%)
1	17	2	11.7
2	51	5	9.8
3	21	2	9.5
4	26	6	23.07
5	32	0	0
6	28	3	10.7
7	26	4	15.3
8	21	1	4.7
9	6	2	33.3
10	12	1	8.3
11	75	7	9.3
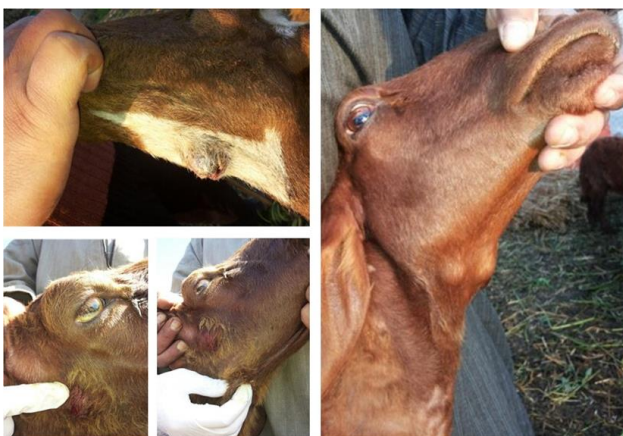
Fig. 1: External lymphadenopathy, characterized by the development of closed abscesses, is a notable clinical feature observed in sheep afflicted with pseudotuberculosis.

Prepared antigens concentration and performance: Based on the previously mentioned antigen quantification steps, the obtained OD values of somatic and exotoxin antigens indicated that their protein concentration was 1.6mg/ml and 5mg/ml, respectively. By comparing the

performance of the prepared somatic and exotoxin antigens against rPLD using ELISA performed on the reference positive and negative sera, there was a strong positive correlation between the tested antigens and the rPLD. The Pearson's correlation between exotoxin and the PLD was 0.927 (P<0.014) while it was 0.821 (P<0.09) between the somatic antigen and PLD. The Pearson's correlation between exotoxin and the somatic antigens was 0.929 (P<0.036). The comparative OD values performance of the three antigens is shown in Fig. 2.

Selection of coating buffer: The correlation between the two used coating ELISA buffers for each prepared antigen to examine their effects on the performance of ELISA was calculated. The Pearson's correlation between Carbonate bicarbonate buffer and PBS for the exotoxin was 0.970 (P<0.015) and for the somatic antigen was 0.919 (P<0.041). Fig. 3 and 4 show the graphic and tabulated illustrations of the correlation between the two buffers for exotoxin and somatic antigens, respectively.

**Comparative performance of serum and milk ELISA:** The correlation between the serum samples, whole milk samples, and milk serum samples was examined, shown in Table 2 and graphically presented in Fig. 5. There was a positive Pearson correlation between the results of serum and whole milk samples of the same animal, but it was not statistically significant; 0.59 (P<0.125). Similar results for the cream-devoid milk and serum; 0.423 (P<0.281). The correlation between both cream-devoid milk samples and whole milk was statistically significant; 0.93 (P<0.001).
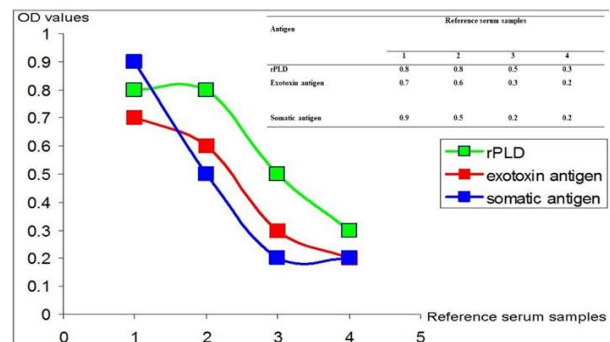
**Fig. 2:** The comparative efficacy of the prepared somatic and exotoxin antigens in relation to rPLD was assessed using two reference positive (1 and 2) and two reference negative (3 and 4) serum samples.
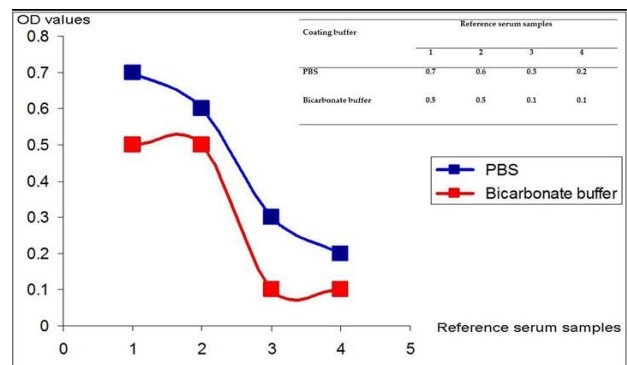
Fig. 3: Performance of PBS and bicarbonate buffer as a coating solution for the prepared exotoxin antigen against two reference positive (1 and 2) and two reference negative (3 and 4) serum samples.
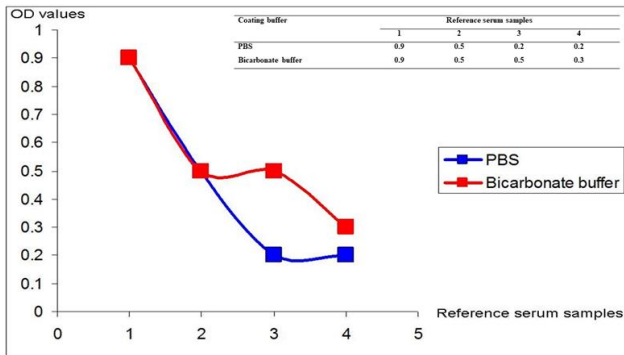
Fig. 4: Performance of PBS and bicarbonate buffer as a coating solution for the prepared somatic antigen against two reference positive (1 and 2) and two reference negative (3 and 4) serum samples.
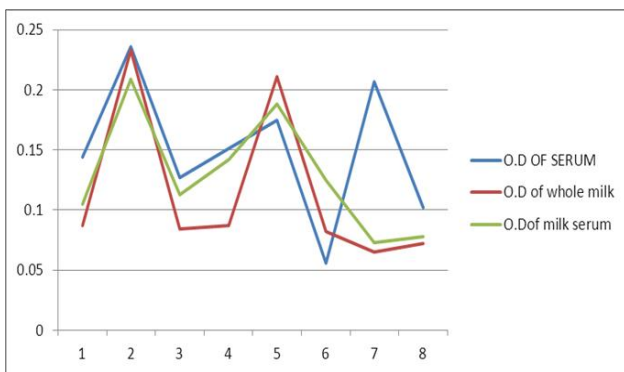
Fig. 5: Correlation between the serum samples, whole milk samples and cream-devoid milk samples of lactating ewes.

Table 2: OD values of serum, whole milk, and cream-devoid milk of lactating ewes.

Animal ID	OD values		
	Serum	Whole milk	Cream-devoid milk
1	0.144	0.087	0.105
2	0.236	0.233	0.209
3	0.127	0.084	0.113
4	0.151	0.087	0.142
5	0.175	0.211	0.188
6	0.56	0.082	0.125
7	0.207	0.065	0.073
8	0.102	0.072	0.078

DISCUSSION

From one health perspective, sheep could be a source for minor zoonoses such as *Corynebacterium pseudotuberculosis* infection or even other pathogens including antimicrobial resistance bacteria (Khalifa *et al.*, 2021). All have negative economic impacts, particularly pseudotuberculosis which reduces wool and meat production as well as the reproductive efficiency of an important animal such as sheep which also has religious importance (Abou Sheasha *et al.*, 2015). Abscess formation is a characteristic lesion of ovine pseudotuberculosis that results from the unique nature of *Corynebacterium pseudotuberculosis*. This pathogen has a lipid-rich cell wall which is responsible for the pyogenic nature of the disease and enables this bacterium to survive intra-cellular digestion of leukocytes. In addition, *Corynebacterium pseudotuberculosis* produces potent exotoxin which is termed phospholipase-D. The toxin is a permeability factor that promotes the dissemination of *Corynebacterium pseudotuberculosis* and its effects on

the functions of immunity cells. Therefore, lesions are found in lymph glands and the infection is persistent which gives the chronic nature of the disease. Variable prevalence rates were previously reported in Egypt as well as in other countries. Such variation is dependent on many overlapping and complex factors (Oreiby *et al.*, 2014b). These factors include but are not limited to animal breed, breeding habits, performed risk practices, degree of endemicity of the disease in the region, stocking density, and biosecurity practices.

The optimal method of control of ovine pseudotuberculosis is through the eradication of infection by identification and removal of infected carrier animals. Since clinical signs cannot identify all infected animals, serological testing detects these unseen cases. Ignoring the control of pseudotuberculosis in sheep will result in a steady increased economic loss. In addition, it will increase the potential risk of human exposure. Creating a cost-effective test which is suitable to be used for screening purposes against this important disease will be of great benefits. It will help to design an insightful control program, which in turn will help in reducing the prevalence of the disease. Consequently, economic losses as well as public health burden resulting from this disease will be reduced.

Many serological tests have been proposed for pseudotuberculosis diagnosis because of the low sensitivity of bacterial isolation and PCR in old lesions besides the lack of clinical signs to capture all infected animals (Baird and Fontaine 2007; Pacheco *et al.*, 2007). ELISA has been proven to be of high sensitivity and specificity for the diagnosis of pseudotuberculosis. Also, it has a low cost and high testing capacity of 90 animals in one plate which offers quick time for testing (Menzies *et al.*, 2004). Several types of ELISA have been developed with different antigen constituents: PLD, somatic antigen…. etc (Paton, 2010; Hassan *et al.*, 2011). However, none are commercially available in Egypt. It was therefore necessary to develop a new, economic, locally produced antigen for use in a research project studying the epidemiology of pseudotuberculosis in Egypt.

The ELISA presented in this study was based on two crude bacterial antigens; one of them contains all the antigens in the cell supernatant (exotoxin) and the other antigen is the sonicated *Corynebacterium pseudotuberculosis* cell antigen. Exotoxins of *Corynebacterium pseudotuberculosis* especially phospholipase-D are a permeability factor that assists the bacterium in the establishment and spread of infection within the host's body. The cell wall is very rich in lipids and protects the bacterium from the lysing effect of intracellular lysozymes. Consequently, exotoxin and cell walls are virulence factors and immunologically important antigens as well. The results of this study showed that there is a strong correlation between the commercial rPLD antigen and both of the prepared antigens. This gave us the confidence to use these antigens for the serological examination of the serum and milk samples. In spite that some authors reported that a cell supernatant (exotoxin) antigen performed better than sonicated cells (somatic antigen) (Paule *et al.*, 2003; Meyer *et al.*, 2005), there was a strong positive significant correlation between both

prepared antigens on the reference serum samples. These results declared that both antigens have a relatively similar diagnostic value to rPLD.

The evaluated diagnostic performance of ELISA tests applied on milk in this study for the first time, up to our knowledge, may be used as a part of a scheme for the surveillance of pseudotuberculosis infections in sheep. Using ELISA on milk for the diagnosis of the disease has not been performed for sheep, and it was used for the first time in goats by Nagel-Alne *et al.* (2015). In this study, we were not able to detect the cut-off specificity and sensitivity of ELISA to be applied to milk. This is because of the lack of control positive and control negative milk samples. In Egypt, sheep are not reared for milk production, therefore, lactating ewes are very limited within sheep herds and only limited to the suckling period of the newly born lambs. Therefore, we tried to compare the results of whole milk and cream-devoid milk with the serum of the same animals. We found that there is a positive correlation between serum ELISA and ELISA results on whole milk and cream-devoid milk. This correlation was not significant, we suggest future work be done with either control positive and negative samples or with suitable number of serum and milk samples of the same animals for accurate estimation of the reliability of this approach. On the other hand, the cream-devoid milk and whole milk results were significantly correlated, and this gives an idea of using either one of them in the future ELISA work, especially the cream-devoid milk as there are sometimes problems with milk fat in serology. Testing of milk using ELISA does not offer a better choice over the serum ELISA as sheep in Egypt are reared mainly for meat production and the percentage of lactating animals in sheep flocks is very small. However, testing of milk samples may offer an easy and realistic way to shepherds. This is because most of them do not like the veterinarian to take blood samples from their animals which causes great disturbance for both the animals and their keeper, and also the bad history of shepherds with brucellosis control programs prevents them from collaborating with these sampling procedures. Dairy sheep herds exist in some countries and such an approach may be of value for them especially if the tank milk is used and the milk ELISA is standardized to identify the status of dairy sheep herds based on single testing of tank milk. The current study contains two limitations; the few numbers of samples tested (eight samples of serum and milk), and the absence of reference milk samples which should be considered in future investigations.

Conclusions: Conclusively, the study revealed fluctuating prevalence rates among the examined flocks, underscoring the necessity for investigating underlying risk factors. The exotoxin and somatic antigens developed in this research demonstrate efficacy as solid phases for ELISA and other serological diagnostics for pseudotuberculosis. Nevertheless, comprehensive investigations involving a substantial number of lactating ewes are imperative to ascertain the precise correlation between serum and milk ELISA outcomes and to validate the reliability of this methodology. Therefore, future studies on a large number of milk samples are required for accurate estimation of the reliability of this approach as there is no dairy sheep production in Egypt.

Authors contributions: Conceptualization, DR, AO, YH, AT, HOK, MAL and TM; Data curation, MA and TM; Formal analysis, AO; Investigation, AO; Methodology, DR, AO and YH; Project administration, MA; Supervision, AO, YH and MA; Validation, DR, AO, YH, AT and TM; Visualization, HOK; Writing – original draft, DR; Writing – review & editing, AO, YH, AT, HOK, MA and TM.

REFERENCES

Abou Sheasha H, Hegazy Y, Oreiby AF, *et al.,* 2015. Encephalitic listeriosis in sheep at Kafrelsheikh Governorate; diagnostic and therapeutic study. Kafrelsheikh Vet Med J 13:49-66.

Aftabuzzaman M and Cho YI, 2021. Recent perspectives on caseous lymphadenitis caused by *Corynebacterium pseudotuberculosis* in goats-A review. Korean J Vet Serv 44(2): 61-71.

Al-Gaabary MH, Osman SA and Oreiby AF, *et al.,* 2009. Caseous lymphadenitis in sheep and goats: Clinical, epidemiological and preventive studies. Small Rumin Res 87:116-121.

Al-Gaabary MH, Osmana SA and Ahmed MS, 2010. Abattoir survey on caseous lymph adenitis in sheep and goats in Tanta, Egypt. Small Rumin Res 94:117-124.

Baazizi R, Mimoune N, Chahed A, *et al.,* 2024. Prevalence and identification of *Corynebacterium pseudotuberculosis* in slaughtered sheep in central Algeria. Veterinarska Stanica 55:289-299.

Baird GJ and Fontaine MC, 2007. *Corynebacterium pseudotuberculosis* and its role in ovine caseous lymphadenitis. J Comp Path 137:179-210.

Binns SH, Green LE and Bailey M, 2007. Development and validation of an ELISA to detect antibodies to *Corynebacterium pseudotuberculosis* sera. Vet Microbiol 123:169-179.

Borham M, Oreiby A, El-Gedawy A, *et al.,* 2017. Caseous lymphadenitis in Sudanese and Somalian camels imported for meat consumption in Egypt. Alex J Vet Sci 55:52-59.

Borham M, Oreiby A, El-Gedawy A, *et al.,* 2021. Abattoir survey of bovine tuberculosis in Tanta, centre of the Nile delta, with *in silico analysis* of gene mutations and protein-protein interactions of the involved mycobacteria. Transbound Emerg Dis 69:434-450.

Borham MA, Oreiby AF, El-Gedawy AA, *et al.,* 2016. Serological surveillance of caseous lymphadenitis in Sudanese and Somali camels slaughtered at Al-warraq Abattoir, Giza, Egypt. World's Vet J 6:89-94.

Bradford MM, 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding a Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing principle of protein-dye binding Anal Biochem 72:248-254.

Di Donato A, Gambi L, Ravaioli V, *et al.,* 2024. First Report of Caseous Lymphadenitis by *Corynebacterium pseudotuberculosis* and Pulmonary Verminosis in a Roe Deer (*Capreolus capreolus* Linnaeus, 1758) in Italy. Animals 14:566.

Dos Santos RM, Marchioro SB, Ribeiro MB, *et al.,* 2022. Assessment of rSodC, rPknG, rNanH, and rSpaC as Antigens for Diagnostic Tools Against Caseous Lymphadenitis. Curr Microbiol 79: 283.

Guimaraes AS, Seyffert N, Bastos BL, *et al.,* 2009. Caseous lymphadenitis in sheep flocks of the state of Minas Gerais, Brazil. Small Rumin Res 87:86-91.

Hassan NA, Al-Humiany AA, Bahobail AS, *et al.,* 2011. Bacteriological and pathological studies on caseous lymphadenitis in sheep in Saudi Arabia. Int J Microbiol Res 2:179-183

Hegazy YM, Abdel-Hamid NH, Eldehiey M, *et al.,* 2022. Trans-species transmission of *Brucellae* among ruminants hampering brucellosis control efforts in Egypt. J Appl Microbiol 132:90-100.

Khalifa HO, Oreiby A, El-Hafeez AAA, *et al.,* 2021. High β-lactam and quinolone resistance of Enterobacteriaceae from the respiratory tract of sheep and goat with respiratory disease. Animals 11:2258.

Menzies PI, Hwang TI and Prescolt JF, 2004. Comparison of an interferon gamma to phospholipase D enzyme-linked immunosorbent assay for diagnosis of *Corynebacterium pseudotuberculosis* infection in experimentally infected goats. Vet Microbiol 100:129- 137.

Meyer R, Regis L, Vale V, *et al.,* 2005. *In vitro* IFN-gamma production by goat blood cells after stimulation with somatic and secreted

Corynebacterium pseudotuberculosis antigens. Vet Immunol Immunopathol 107:249-254.

Nagel-Alne GE, Valle PS, Krontveit R, *et al.,* 2015. Caprine arthritis encephalitis and caseous lymphadenitis in goats: use of bulk tank milk ELISA for herd-level surveillance. Vet Rec 176:173.

Oreiby AF, 2015. Diagnosis of caseous lymphadenitis in sheep and goat. Small Rumin Res 123: 160-166.

Oreiby AF, Abo El-Wafa SA, Hegazy YM, *et al.,* 2014a. Oedematous skin disease, a comparison of changeable clinical nature in cattle and buffalo. Kafrelsheikh Vet Med J 12:91-108.

Oreiby AF, Hegazy YM, Osman SA, *et al.,* 2014b. Caseous lymphadenitis in small ruminants in Egypt. Tierärztliche Praxis Ausgabe G: Großtiere/Nutztiere 42:271-277.

Oreiby AF and Hegazy YM, 2016. Diagnosis of ovine caseous lymphadenitis by blood and milk gamma interferon assays. Small Rumin Res 144:109–112.

Oreiby AF, Osman SA, Hegazy Y, *et al.,* 2013. Caseous lymphadenitis in small ruminants: Descriptive, epidemiological and clinical studies. Kafrelsheikh Vet Med J 11: 41–61.

Pacheco LGC, Pena RR, Castro TLP, *et al.,* 2007. Multiplex PCR assay for identification of *Corynebacterium pseudotuberculosis* from pure cultures and for rapid detection of this pathogen in clinical samples. J Med Microbiol 56:480–486.

Paton MW, 2010. The epidemiology and control of Caseous Lymphadenitis in Australian sheep flocks. Ph. D. Thesis, School of Veterinary and Biochemical Sciences. Murdoch University.

Paule BJA, Azevedo V, Regis LF, *et al.,* 2003. Experimental *Corynebacterium pseudotuberculosis* primary infection in goats: kinetics of IgG and interferon production IgG avidity and antigen recognition by Western Blotting. Vet Immunol Immunopathol 96:129–139.

Rizk DY, Hegazy YM, Oreiby AF, *et al.,* 2015. Some epidemiological and preventive measures for caseous lymphadenitis in sheep at Qualin district. Kafrelsheikh Vet Med J 13:1-16.

Tahoun A, Jensen K, Corripio-Miyar Y, *et al.,* 2015. Functional analysis of bovine TLR5 and association with IgA responses of cattle following systemic immunisation with H7 flagella. Vet Res 46:1-15