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Bacteriological and Biotechnological Studies on *Corynebacterium Pseudotuberculosis* in ovine; Detection of *PLD* gene by PCR

Amany, M. Rafeque*; EL.Toukhy, E. I.; Ola, A. Abd El Fattah*** and Hanaa, A. Ahmed****

* Fayoum Lab., ** Biotechnology Research Department/Dokki, *** Giza Lab., Animal Health Research Institute, Egypt

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Abstract

This study was carried at El Fayoum governorate; five isolates out of 26 sample of pus and lymph nodes from balady sheep were detected by direct isolation which identified as *C. pseudotuberculosis* and two of them confirmed by Polymerase Chain Reaction (PCR) at 816 bp of the *16S rRNA* gene, also all isolates produce phopholipase D at 203 bp band of PLD gene. Pathogenesis of *C. pseudotuberculosis* was assayed by S/C inoculation in five guinea pigs then further reisolation and identification was carried.

Key words: *Corynebacterium pseudotuberculosis, Phopholipase D (PLD), PCR, CLA.*

Introduction

Caseous lymphadenitis (CLA) is a chronic and subclinical disease that affects goats and sheep and, consequently, causes economic losses, especially to small producers **(Aquino *et al*., 2013).** A group of potentially toxigenic microorganisms including *Corynebacterium diphtheriae*, *Corynebacterium ulcerans* and *Corynebacterium pseudotuberculosis* are related to different infectious processes in animal and human hosts. Currently, little information is known on the prevalence of disease caused by these pathogens that is partially due to a reduction in the frequency of routine laboratory testing **(Torres *et al*., 2013).** *C. pseudotuberculosis* and *C. ulcerans* are differentiated from *C. dipheriae* by their production of exotoxin phospholipase D (PLD) (**Barksdale, 1981**).

C. pseudotuberculosis is the causative agent of caseous lymphadenitis (CLA) in small ruminants that sometimes presented as pneumonia, hepatitis, pericarditis, mastitis, arthritis and subcutaneous abscesses; (OSD) oedmatous skin disease in buffaloes (**Selim, 2001** and **Sharpe *et al*., 2010).** In Egypt both disease are of opposite characteristic, while CLA is of chronic nature always associated with development of abscesses discharging cheesy pus infecting external lymph nodes and internal organs (**PointKowski *et al*., 1998**).

The potential of *C. pseudotuberculosis* to survive for several weeks in the environment likely contributes to its ability to spread within a herd or flock (**Augustine and Renshaw, 1986and Yeruham *et al*., 2004)**. Transmission among sheep or goats occurs mainly through contamination of superficial wounds, which can appear during common procedures, such as shearing, castration and ear tagging or through injuries of the animals' bodies generated by other traumatic events **(Williamson, 2001).**

The pathogenesis of *C. pseudotuberculosis* is due to two major virulence factors; one of these is the toxic lipid cell wall that resists killing by host phagocyte (**Tashjian and Campbell, 1983**) and therefore maintains as facultative intracellular organism. This thick wall lipid resists antimicrobial treatment; therefore, vac-

cination would be helpful in disease control (**PointKowski, *et al*., 1998**). The second main virulence component of *C. pseudotuberculosis* is a sphingomyclin – degrading exotexin called phospholipase D (PLD). There is a clear empirical evidence that PLD is a major virulence factor as it mediates the dissemination of pathogen within the host by increasing the local vascular permeability **(Batey, 1986).**

The present work was carried out to diagnose some cases of CLA in sheep bacteriologically and confirm it with Polymerase Chain Reaction (PCR) together with detection of the virulence gene controlling the formation of Phospholipase D (PLD).

Material and Method

Specimens.

Pus samples (Swabs) were collected aseptically from 16 living balady sheep (1–3 years old) showing closed abscessed superficial lymph nodes suspected to be caseous lymphadenitis.

Ten lymph nodes obtained from balady sheep (1–2 years old) that were slaughtered at El Fayoum abattoir. The collected lymph nodes included (prescapular, submundibular, prefemoral, tracheal, bronchial, hepatic, spleenic and mesenteric L.N.) showing dough enlargement. All samples (swabs and L.N) were aseptically collected and transferred in an icebox to the laboratory.

C. pseudotuberculosis isolation and identification.

The surface of the infected lymph nodes samples were cleaned with 70% ethanol, left for dryness and incised with sterile scalpel. The inner caseated material was squeezed out and swabs were collected from the inner parts of the lymph nodes (**Barakat *et al*., 1984).** Swabs were streaked onto the surface of brain heart infusion agar (BHIA) supplemented with 200 mg fosfomycin and 4 mg nalidixic acid then incubated for 48 hrs at 37°C. *C. pseudotuberculosis*-resembling colonies that stained Gram-positive were tested further for biochemical properties (glucose fermentation, urease, nitrate **(Eggleton *et al*., 1991)**. Synergistic haemolysis with *Rhodococus equi* ATCC33701 and inhibition of B-haemolysis by *Staphylococcus aureus* ATCC25923 were also evaluated according to **(Pépin *et al*., 1989).**

Synergistic hemolytic activity of *C.pseudotuberculosis* with *Rhodoccus equi* filtrate by streaking into L.B agar containing 5% sheep erythrocytes and 10% *R.equi* filtrate and observe the zone of hemolysis which surround the tested colonies. Inhibition of B-hemolysis by *Staphylococcus aureus* with PLD was applied by reverse CAMP test.

Pathogenisity test of *C. pseudotuberculosis* isolated from sheep according to Muckle and Gyles (1983). 1×10^6 bacterial cell counts were inoculated subcutaneously in 5 guinea pig and 1 gunia pig as control. The inoculated guinea pigs were observed daily for 15 days. Mortality guinea pigs were examined to detect the changes in site of inoculation and lesion in the internal viscera. Resolution from abscesses and lesion of internal organs (liver, spleen) was performed.

DNA Extraction. using the rapid boiling method **(Bansal, 1996).** Bacterial pellets of 48-72 h incubation of cultured isolates were washed once with 200-µl phosphate buffered saline (PBS), pH 7.4, re suspended in 50 µl PBS and incubated in a boiling water bath for 10 min., then in freeze for another 10 min. The clear supernatants obtained after a 5 min centrifugation at 12000g were used for PCR reaction.

PCR is done for confirmation of the isolation using the *16S rRNA* gene **(Cetinkaya *et al*., 2002)**, then detection of *PLD* gene **(Pacheco *et al*., 2007)** as virulence gene according to table (1).

Gene	Primers (5' to 3')	Thermal profile	Product size
16S rRNA	16S-F ACCGCACTTTAG-TGTGTGTG 16S-R TCTCTACGCCGATCTT-GTAT	Initial denaturating step at 94 ^{o}C for 5 min. followed by 30 cycles of denaturation at 94 ^{o}C for1 min, annealing at 56 ^{o}C for 1 min, and synthesis at 72 ^{o}C for 2 min. then final extension for 10 min at 72 ^{o}C.	816 bp
PLD gene	PLD-F ATAAGCGTAA-GCAGGGAGCA PLD-R1 ATCAGCGGTGATTGTCTTCC	Initial denaturation at 95 ^{o}C for 3 min; 40 cycles of 95 ^{o}C for 1 min, 58 ^{o}C for 40 s and 72 ^{o}C for 1 min 30 s; final extension at 72 ^{o}C for 7 min.	203 bp

The amplified products were detected by ethidium bromide (0.5 mg/ml) staining after electrophoresis at 60–70 V for 1-2 h in 1.5% agarose gels.

Results and Discussion

Caseous lymphadenitis (CLA) is a chronic disease that affects sheep and goats consequently causes economic losses, especially to small producers **(Aquino *et al*., 2013).** This study was performed in different districted areas of El Fayoum governorate. The clinical signs of abscesses in L.N. and internal organs were nearly the same recorded by **(Point Kowski *et al*., 1998).**

Table (1). Occurrence of isolated *C.pseudotuberculosis* in examined infected sheep and L.N.

Type of sample	Total number of sample	Results			
		Negative		Positive	
		No	%	No	%
lymph nodes	10	9	90	1	10
Swabs	16	12	75	4	25

Table (1) showed that five isolates of *C. pseudotuberculosis* were detected, 4 isolates (25%) from the superficial L.N. (prescapular and prefemoral) and one isolate (10%) was detected from internal L.N. (hepatic, mesenteric,splenic, tracheal and bronchial), these results were in agreement with that of **(Barakat *et al*., 1984)** ; **(Soheir, 2002)** and **(Selim *et al*.,**

Identification of *C.pseudotuberculosis* isolates.

Table (2). The results of biochemical activities of *C. pseudotuberculosis* isolated from sheep.

Test Isolate number	Cataloes		Nitrate		Urease		Starch		Glucose		Xylose		Manitel		Lactose		Glatose		Fructose		Trehalose		Sucrose	
5	+ve	- ve	+ve	- ve	+ve	- ve	+ve	- ve	+ve	- ve	+ve	- ve	+ve	- ve	+ve	- ve	+ve	- ve	+ve	- ve	+ve	- ve	+ve	- ve
	5	0	0	5	5	0	0	5	5	0	0	5	0	5	0	5	0	5	5	0	4	1	5	0

The pattern of diagnostic tests for 5 strains of *C.pseudotuberculosis* of sheep were illustrated in Table 2 revealed that nitrate reaction test was negative and urease positive and other biochemical character of sugar fermentation tests.

Synergistic hemolytic activity of exotoxin (PLD) of *C.pseudotuberculosis* and *R.equi* filtrate represented by clear zone of hemolysis surrounded the tested colonies, the diameters of intensity of which depend on the extent of toxin production of the isolate. All isolates (5strains) had a synergistic hemolytic activity of PLD with *R.equi* **(Photo.1**).

Photo. (2), showed inhibition of B-hemolysis by staphylococcus aureus with PLD reverse CAMP test. The exotoxin producer stain of *C. pseudotuberculosis* inhibited the hemolytic activity of (vertical streaked colonies) B-hemolysin produced by staphylococci that appear as arrow shape between the two streaked strains. All corynebacterial sheep isolates (100%) were inhibitors for B-hemolytic staphylococcal hemolytic activity **(Songer *et al.*, 1990)**

Pathogensity test.

All isolates developed abscess at site of inoculation with 1×10^6 bacterial cell count. Lethality (100%) occurred after 4 – 10 days. Postmortem lesion included abscess in the internal viscera, these results are in agreement with **(Muckle and Gyles 1983); (Soheir, 2002) and (Selim, *et al.,* 2012).** Reisolation of microorganism from internal organs of dead guinea pigs, these results are similar with that recorded by **(Hala, 2009). Hard (1975)** contributed the hemorrhagic necrosis after intradermal injection in guinea pigs to the surface lipid components of *C. pseudotuberculosis* during pathogenesis

The isolation of *C. pseudotuberculosis* was confirmed in some isolates by detecting the *16S rRNA* gene that produced 815 bp (**Photo. 3**). These results matched with the findings of ***(Cetinkaya et al., 2002); (Pacheco et al., 2007) and (Sohier et al., 2013).***

Phospholipase D (*PLD*) is well established as the important virulence factor of *C. pseudotuberculosis* **(Selim *et.al.*, 2012).** This gene is the major virulence factor of *C. pseudotuberculosis* **(Hodgson *et al*., 1999).** It encodes an exotoxin that promotes the bacterial dissemination, increasing vascular per-meability through the hydro-lysis of ester bonds in sphingomyelin of mammalian cell membranes following infection **(Dorella *et al*., 2006),** enabling the bacteria to escape from neutrophils, and impairing neutrophil chemotaxis toward the site of infection **(Yozwiak and Songer, 1993).**

Cetinkaya et. al. (2002) reported that the availability of a *C. pseudotuberculosis*-specific PCR might be useful on several occasions. First, it might be used as an aid in the identification of cultured organisms, since final biochemical identification may not always be straightforward. Second, it might be useful as a sensitive, specific assay for direct detection of *C. pseudotuberculosis* and as such be used as an aid in vaccination studies.

Photo. (4), represents Phospholipase D (PLD) gene that produced a specific amplicone size of

204 bp. These findings were nearly similar with that of **(Songer *et al.*, 1988); (Soheir, 2002) and (Selim et al., 2012).** This result is the same discussed by **(*Pacheco et al., 2007*)** and **(Ilhan, 2013).**

It was believed that this gene is not enough to confirm *C. pseudotuberculosis* as this region is also similar to that of *C.ulcerans.* **(Barksdale *et al.*, 1981)** reported that phospholipase D activity seems to be the same in those two species than other species of *Corynebacterium*, however *C. ulcerans* did not reported in small ruminants.

Rapid and accurate detection of potentially toxigenic corynebacteria is necessary for the determination of appropriate measures in controlling the dissemination of toxigenic clones in both veterinary and/or human populations. PCR assays offer many advantages over standard phenotypic tests: they are simple, easy interpreted, and becoming more widely available in laboratories in both industrialized and in developing countries **(Torres *et al*., 2013).**

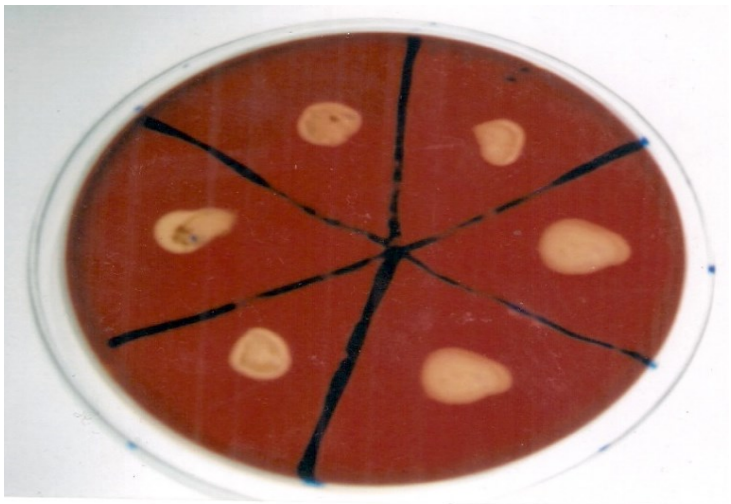

Photo (1). Synergisitic haemolytic activity of *C. pseudotuberculosis* with *R.equi* filtrates.

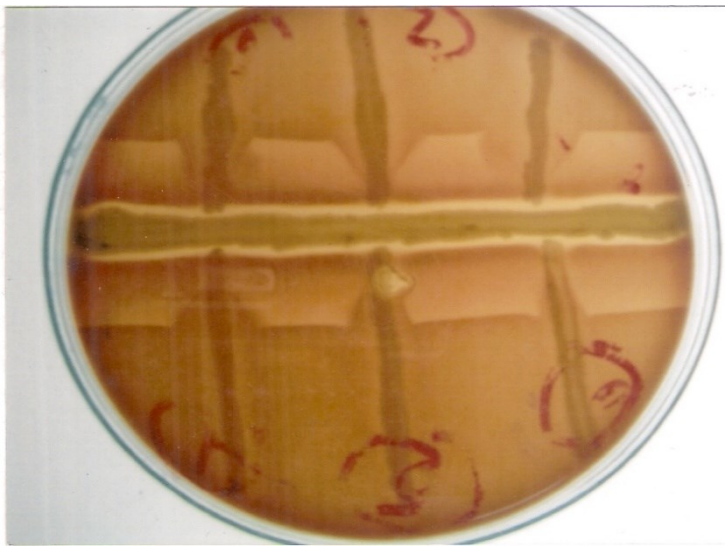

Photo (2). Inhibition of staphylococcal hemolytic activity with PLD (reverse CAMP test)

M- molecular marker (Fermentas)
1.*C. pseudotuberculosis isolate 1*
2.*C. pseudotuberculosis isolate 2*
3.Control negative

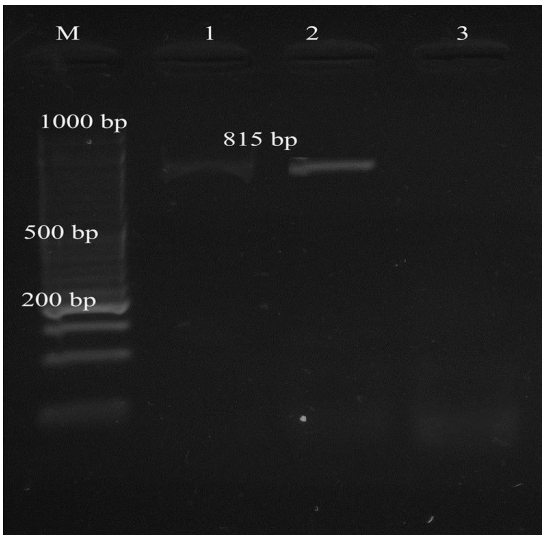

Photo (3). Show the electrophoretic pattern of the 16S rRNA gene of *C. pseudotuberculosis* isolates

M- molecular marker (Fermentas)
1.*C. pseudotuberculosis* isolate 1
2.*C. pseudotuberculosis* isolate 2
3.Control negative

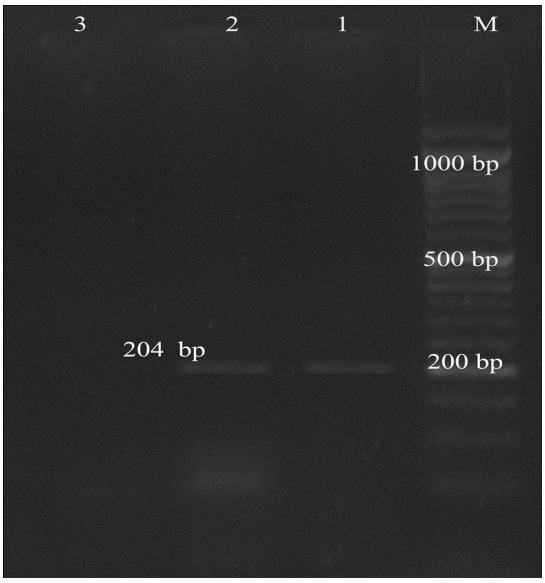

Photo (4). Show the electrophoretic pattern of the *PLD* gene of *C. pseudotuberculosis* isolates.

In conclusion, Phospholypase D produced by *C.pseudotuberculosis* plays an important role of the virulence factor that induces caseous lymphadenitis infection in sheep, molecular diagnosis can help in rapid and sensitive detection of it. Interests should be directed to the health hazard that arises from this important zoonotic microbe. Further studies on virulence factors genes of *C.pseudotuberculosis* are in need to control CLA disease through vaccination.

References

Aquino, D.C., Gouveia, G.V., Krewer, C., Veschi, L., de Mattos-Guaraldi, A. and da Costa, M.M. (2013). Distribution of PLD and FagA, B, C and D genes in *Corynebacterium pseudotuberculosis* isolates from sheep and goats with caseus lymphadenitis. Genet. Mol. Biol., 36(2):265-268.

Augustine, J.L., Renshaw, H.W. (1986). Survival of *Corynebacterium pseudotuberculosis* in axenic purulent exudate on common barnyard fomites Am J Vet Res., 47(4):713-715.

Bansal, N.S. (1996). Development of a polymerase chain reaction assay for the detection of *Listeria monocytogenes* in foods. Letters of Applied Microbiology, 22:353–356.

Barakat, A.A., Selim, S.A., Atef, A., Saber, M.S., Nafie, E.K. and El-ebeedy, A.A. (1984). Two serotypes of *corynebacterium pseudotuberculosis* isolated from different animal species. Rev. Sci. Tech. off. Int. Epiz., 3 (1): 151 – 168.

Barksdale, L. (1981). The genus *corynebocterium* in: starr, M.P., II stolp, II.G. Truper, A. Bulows and IIG. Schlegel, geds. The parokaryotes; II: 1827 – 1839, Springer rerlay, new York.

Batey, R.G. (1986). Pathogenesis of gaseous lymphadenitis in sheep and goats. Rust. Vet. J., 63 (9): 269 – 292.

Cetinkaya, B., Karahan, M., Atil, E., Kalin, R., De Baere, T. and Vaneechoutte M. (2002). Identification of *Corynebacterium pseudotuberculosis* isolates from sheep and goats by PCR. Vet. Microbiol., 2;88(1):75-83.

Dorella, F.A., Pacheco, L.G., Oliveira, S.C., Miyoshi A. (2006). *Corynebacterium pseudotuberculosis*: microbiology, biochemical properties, pathogenesis and molecular studies of virulence. Vet. Res., 37: 201-218.

Eggleton, D.G; Middleton, H.D.; Doldge, C.V. and Minty, D.W. (1991). Immunisation against ovine caseous lymphadenitis, comparison of Corynebacterium pseudotuberculosis vaccines with and without bacterial cells. Aust. Vet. J., 68 (10):317-319.

Hala, R.K. Aly (2009). Identification of proteins produced by *C. pseudotuberculosis* other than PLD. M.V.S.c. Thesis, Fac. Vet. Med., Cairo Univ.

Hard GC (1975). Comparative toxic effect of the surface lipid of *Corynebacterium ovis* on peritoneal macrophages. Infect. Immun., 12 (6):1439-1449

Hodgson AL, Carter K, Tachedjian M, Krywult J. (1999). Efficacy of an ovine caseous lymphadenitis vaccine formulated using a genetically inactive form of the *Corynebacterium pseudotuberculosis* phospholipase D. Vaccine, 17: 802-808.

Ilhan, Z. (2013). Detection of *Corynebacterium pseudotuberculosis* from sheep lymph nodes by PCR. Revue Méd. Vét., 164(2): 60-66.

Muckle, C.A. and Gyles, C.L. (1983). Relation of lipid content and exotoxine production to virulence of *C. pseudotuberculosis* in mice. Am.J.Vet.Res., 44(6):1149-1153.

Pacheco LG, Pena RR, Castro TL, Dorella FA, Bahia RC, Carminati R, Frota MN, Oliveira SC, Meyer R, Alves FS, Miyoshi A, Azevedo V. (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(Pt 4):480-486.

Pépin, M., Boisramé, A. and Marly, J. (1989). *Corynebacterium pseudotuberculosis* : biochemical properties, production of toxin and virulence of ovine and caprine strains. Ann Rech. Vet., 20(1):111-115.

Piontkowski, M.D., Micheal D.M. and Shivers, D.W. (1998). Evaluation of a commercially available vaccine against *corynebacterium pseudotuberculosis* for use in sheep. J. Am. Vet. Med. Assoc., 212 (11): 1765 - 1768.

Selim, S.A., Mousa, W.M., Mohamed, K.F. and Moussa, L.M. (2012). Synergistic haemolytic activity and its correlation to phospholipase D productivity by *corynebacterium*

pseudotuberculosis Egyptian isolation from sheep and buffaloes. Brazilian J. of Microbiology,552-559.

Selim, S.A. (2001). Edematous skin disease of buffalo in Egypt: a review. J. Vet. Med. B., 241 – 258.

Sharpe, A.E., Brady, C.P., Johnson, A.J., Byrne, W., Kenny, K. and Costello, E. (2010). Concurrent outbreak of tuberculosis and caseous lymphadenitis in a goat herd. Vet. Rec., 166: 591-592.

Soheir, M.F.S (2002). Incidence of *corynebacterium pseudotuberculosis* in different animals sheep, goat and buffaloes. M. V. Sc., Microbiol. Depart. Fac. Vet. Med., Cairo Univ., Giza Egypt.

Sohier M. Syame, Hakim, A.S. , Riham, H., Hanan, S..H and Selim, S.A. (2013). Characterization of Virulence Genes Present in *Corynebacterium pseudotuberculosis* Strains Isolated From Buffaloes. Global Veterinaria, 10 (5): 585-591.

Songer, J.G, Beckenbach, K., Marshall, M.M., Olson, G.B. and Kelly, L. (1988): Biochemical and genetic characterization of *C. pseudotuberculosis*. Am.J.Vet.Res.49 (2): 223-226.

Songer, J.G., Libby, S.J., Iandolo, J.J. and Cuevas, W.A. (1990). Cloning and expression of the *phospholipase D* gene from *Corynebacterium pseudotuberculosis* in *Escherichia coli*. Infect Immun., 58(1): 131-136.

Tashjian, J.T. and Campbell, S.G. (1983). Interaction between capriue macrophages and *corynebacterium pseudotuberculosis*: An electron microscopic study. Am. J. Vet. Res. 44 (4): 690 – 693.

Torres, Lde F, Ribeiro D, Hirata R Jr, Pacheco LG, Souza MC, dos Santos LS, dos Santos CS, Salah M, da Costa MM, Ribeiro MG, Selim SA, Azevedo VA, Mattos-Guaraldi AL. (2013). Multiplex polymerase chain reaction to identify and determine the toxigenicity *of Corynebacterium* spp with zoonotic potential and an overview of human and animal infections. Mem Inst Oswaldo Cruz., 108(3):8-14

Williamson, L.H. (2001). Caseous lymphadenitis in small ruminants. Vet. Clin. North Am. Food Anim. Pract. 17:359–371.

Yeruham, I., Friedman, S., Perl, S., Elad, D., Berkovich, Y. and Kalgard, Y. (2004). A herd level analysis of a *Corynebacterium pseudotuberculosis* outbreak in a dairy cattle herd. Vet. Dermatol., 15:315–320

Yozwiak, M.L. and Songer, J.G. (1993). Effect of *C. pseudotuberculosis* phospholipase D on viability and chemotactic responses of ovine neutrophils. Am. J. Vet. Res., 54: 392-397