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Optimization of a Conventional PCR Assay for the Identification of *Corynebacterium Pseudotuberculosis* from Pyogenic Lesions

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Abstract

Caseous lymphadenitis (CL) is a pyogenic disease that is caused by zoonotic bacteria and is in the same family as *Corynebacterium diphtheriae* (the etiologic agent of human Diphtheria). CL mainly affects small ruminants but has also been described in deer, cows, horses, pigs and humans. The main objective of this study was to optimize conventional PCR (Polymerase Chain Reaction) for the identification of the CL etiologic agent, *Corynebacterium pseudotuberculosis*, from pyogenic lesions. The present work is also a preliminary study of CL prevalence conducted in a slaughterhouse in 335 animals (311 sheep and 24 goats) from 114 herds of small ruminants in the region of Alto Alentejo, Portugal affected by CL. The prevalence of CL was determined by macroscopic observation of the lesions and conventional PCR in pathological samples of pus and affected organs collected from sheep and goats that had external or visceral lesions. The PCR technique for the detection of *Corynebacterium pseudotuberculosis* genomic DNA (as described by Pacheco *et al.* was based on the design of primer pairs for two target genes, the 16S ribosomal segment (16s rRNA) – 815 bp (base pairs) and the gene which encodes the exotoxin Phospholipase D (PLD) – 132 bp both at normal and diluted concentrations. From the gross detection of the lesions the results shows a CL prevalence of 17, 31% (58/335) and indicate a CL prevalence of 17, 24% in affected animals, with the identification of *C. pseudotuberculosis* in 10 of the 58 lesions sampled. The dilution of DNA samples and detection of genomic DNA of *C. pseudotuberculosis* from two different DNA regions (i.e., 16s and PLD) conducted in this investigation allowed maximal agent identification. This is the first report of CL prevalence in Portugal. We conclude that conventional PCR is an excellent tool for definitive and differential diagnosis of CL but is not useful in prevalence studies since it does not allow the identification of subclinical cases.

Keywords: Caseous Lymphadenitis; PCR; Small Ruminants; Pyogenic; Portugal

Article Summary

Extraction and purification of genomic DNA for the detection of *Corynebacterium pseudotuberculosis* are often hampered by the large pyogenic component of typical pathological samples and the high concentration of cellular debris. There has not been sufficient validation of the performance of existing PCR assays for the identification of *C. pseudotuberculosis* from pyogenic lesions. The present work aimed to improve the effectiveness of conventional PCR for the direct detection of *C. pseudotuberculosis* in field samples. Caseous lymphadenitis (CL) is a pyogenic disease with two recognized clinical manifestations: subcutaneous and visceral. The visceral form is primarily responsible for disease dissemination in animal flocks, largely due to the presence of subclinically infected animals. Examination of a slaughterhouse allowed for a real evaluation of CL on farms through the detection of subclinically infected animals. The present work sought to optimize conventional PCR analysis for the identification of *C. pseudotuberculosis* in pyogenic lesions and then apply the new assay to estimate the prevalence of CL in affected small ruminant flocks in Alto Alentejo, a region of the Iberian Peninsula with important sheep and goat livestock production. To our knowledge, this is the first prevalence study of CL in small ruminants carried out in Portugal and the high prevalence found in this study (17%) justifies the implementation of new sanitary measures and suitable control programs to prevent the transmission and dispersion of *C. pseudotuberculosis* in Portugal. This study has certain limitations, including an inability to identify other pathogenic agents in pyogenic lesions, which would have increased clinical knowledge regarding differential diagnoses for CL. The fact that this study included samples of superficial, visceral, acute and chronic lesions, most of which had a strong purulent component, may explain the difficulty associated with the digestion and extraction of genomic DNA from lesions. In addition, false negative results may

have occurred in the case of sterile CL lesions that do not include a bacterial agent or its DNA. These considerations may have led to an underestimation of the true CL prevalence.

Introduction

Caseous lymphadenitis (CL) is a chronic and insidious disease that mainly affects small ruminants. CL is caused by *Corynebacterium pseudotuberculosis*, a Gram-positive bacterium from the actinomyces group of microorganisms [1]. *C. pseudotuberculosis* is a robust microorganism in the external environment, which has largely been attributed to the presence of a lipid component in its external membrane that protects the bacterium very effectively from desiccation [2].

Clinical signs of CL include enlargement of superficial lymph nodes, such as the submandibular, pre-scapular, pre-femoral, popliteal and supra-mammary lymph nodes, as well as the presence of lesions in internal organs, such as the lungs, liver, kidneys and others [3]. The disease is characterized by necrotizing and suppurative inflammation of one or more nodes with features of a necrotic-purulent nature, as well as damage to multiple organs, the lesions of which usually acquire a caseous nature [2].

The true prevalence of CL in small ruminants remains underestimated, and there are no data in Portugal concerning either the prevalence or the economic impact of this disease. CL has been disseminated worldwide, and its economic impact in sheep and goat flocks is a long-standing concern of governmental agricultural departments in developed countries. This has led to the recent implementation of novel control programmes. In addition to its sanitary and zoonotic importance, CL is a major cause of economic losses in sheep and goat flocks worldwide, causing highly impactful disruptions in the production of milk, meat and wool [4-7].

Corynebacterium pseudotuberculosis primarily affects small ruminants, although it has occasionally been found in cattle, horses, buffalo, deer and humans [8-12]. *Corynebacterium pseudotuberculosis* infection in horses is widespread worldwide and may lead to varying clinical conditions [13,14]. In Portugal, the first case of CL in a Black Alentejano pig (*Sus scrofa domesticus*) was reported in 2014, with phenotypic and genotypic characterization of *Corynebacterium pseudotuberculosis* from clinical isolates [15].

The pathogenicity of *C. pseudotuberculosis* strains is related to the secretion of toxic factors, such as Phospholipase D (PLD), and the lipid contents of their cell walls [10,12,16,17].

The transmission of *C. pseudotuberculosis* between infected and healthy animals occurs through direct contact with purulent material and through the inhalation of infected particles [11,18,19].

It has been reported in the United States (USA), Israel and Egypt that *C. pseudotuberculosis* can be dispersed among animals by insect vectors, such as flies (e.g., *Musca domestica and Hippobosca equine*) [12,20]. Numerous studies performed around the world regarding CL control are unanimous in reporting the importance of detecting subclinical animals, a major focus of infection in every affected herd.

There is no single diagnostic test that is able to identify all cases, different phases or forms of the disease [21]. Serological diagnosis based on ELISA (Enzyme-linked Immunosorbent Assay) tests continues to be improved and validated for the identification of subclinical animals [22-25]. However, the definitive diagnosis of CL currently still relies on the isolation and identification of the etiologic agent through biochemical tests, bacterial culture, and, most recently, molecular diagnostic tests including PCR [19,26]. PCR offers a robust technique that has been adopted for the direct detection of microorganisms in a wide variety of clinical and *post-mortem* specimens [26]. However, to date, the effectiveness of PCR for the direct detection of *C. pseudotuberculosis* in pyogenic samples has been poorly evaluated, and there have not been sufficient validation studies regarding the performance of existing PCR tests [27].

The present study sought to optimize conventional PCR techniques for the identification of *C. pseudotuberculosis* in pyogenic lesions, and then apply the newly developed assay to estimate the prevalence of CL in affected flocks of small ruminants in Alto Alentejo, a region of the Iberian Peninsula with important livestock production of sheep and goats. The continental region of Portugal has a total of 2 218 000 sheep and 326 000 goats and almost half of this herd is in the province of Alentejo (1 324 000 sheep and 108 000 goats) (Portugal, 2017). In Alto Alentejo a sub-region of Alentejo there are approximately 250 000 sheep and 87 000 goats. In this sub-region of Portugal the majority of the farms are meat production units in extensive regime, and some in intensive production, being these mainly for milk production. The approximate average number of animals per herds is 114, with farms with 10 to 1200 animals per holding.

Materials and Methods

Animals and Samples

This study was carried out in 114 small ruminant farms (74 sheep farms and 40 goats farms) based in the Portuguese region of Alto Alentejo, located in the district of Portalegre, with different types of production systems, including intensive, semi-extensive and mostly extensive. A total of 335 animals (311 sheep and 24 goats) were observed in a local slaughterhouse to investigate lesions compatible with *C. pseudotubeculosis* infection. From a macroscopic analysis, 17, 31% of the animals (58/335) were identified with

lesions suggestive of CL, which were collected and analyzed (Table 1). Clinical samples (58) of purulent and pyogranulomatous lesions were obtained from visceral lesions and/or internal organs manifesting macroscopic changes suggestive of pyogenic processes, as well as from superficial lesions characteristic of CL.

	Sheep Number (%)	Goats Number (%)	Total
Herds	74 (65)	40 (35)	114
Animals	311 (92, 8)	24 (7, 2)	335
Purulent/pyogranulomatous lesions analyzed	54 (93, 1)	4(6, 9)	58

Table 1: Animals and Samples

The samples were labelled with the location of origin, animal species and type of organ/affected area and preserved by freezing at -20 °C. A total of 58 samples were collected and preserved, which corresponded to one sample per animal.

The preparation of samples for processing by conventional PCR assay was realized by sectioning, with the aid of a scalpel blade, 25 to 30 mg of tissue from the most representative portion of the pathological sample (including purulent characteristic lesions) and placing the sample in a 1.5 ml Eppendorf tube. During the study, we verified that the sample selected for processing included original tissue of the organ or peripheral material of the lesion, making sure that the piece was not sterile and that it contained genomic material of the etiologic agent.

Conventional PCR Method

Primer Design: The DNA from each sample was tested by PCR according to the method described by Pacheco *et al*. The PCR technique for the detection of *Corynebacterium pseudotuberculosis* genomic DNA was based on the design of primer pairs for two target genes, the 16S ribosomal segment (16s rRNA) – 815 bp (base pairs) and the gene which encodes the exotoxin Phospholipase D (PLD) – 132 bp both at normal and diluted concentrations (Table 2).

PCR I	Detection of 16S ribosomal RNA gene (normal DNA concentration)
PCR II	Detection of exotoxin Phospholipase D gene (PLD) (normal DNA concentration)
PCR III	Detection of 16S ribosomal RNA gene (diluted DNA 1:10)
PCR IV	Detection of exotoxin Phospholipase D gene (PLD) (diluted DNA 1:10)

Table 2: Description of PCR assay design for analysis of each of the pathological samples

All primers were designed using the software "Primer Express" (Primer Express™ Software v3.0.1 License) to determine the optimal sequence of nucleotides for both the 16s and PLD segments.

The choice of nucleotide sequence was made using www.ncbi.nlm.nih.gov/nuccore/ as follows:

Primers for the Corynebacterium pseudotuberculosis 16S ribosomal RNA gene:

Fw: GTCTAATACTGGATAGGACCGCACT (58 °C)

Rv: ATATGTCAAGCCCAGGTAAGGTTC (59 °C)
Amplicon: 815 bp
Seq ref: NR_119175.1

Primers for the Corynebacterium pseudotuberculosis PLD gene:

Fw: GCTCGTAGTGTGTGCTCCATAAAT (58 °C)
Rv: ATCAGCGGTGATTGTCTTCCA (59 °C)
Amplicon: 132 bp
Seq ref: L16587.1

DNA Extraction and Digestion: For the DNA digestion and extraction procedure, we used the "NZY Tissue gDNA Isolation kit" from the Portuguese company "Nzytech - genes & enzymes Company" Lisbon, Portugal - Catalogue numbers: MB13502, 50 columns, MB13503, 200 columns, Batch 14071. The NZY Tissue gDNA Isolation kit is optimized for isolating up to 35 µg of DNA from up to 25 mg of tissue or 107 of cells.

In this particular study, the purification of DNA from purulent material proved to be a difficult procedure, resulting in subsequent decreases in test sensitivity.

Agarose Gel Electrophoresis: The obtained bands were confirmed, analyzed and compared to bands obtained from standards for each isolate, and the results were imaged for future reference and analysis.

Statistical analysis of the observed results use SPSS (Statistical Package for Social Sciences) 9.0 and $\chi 2$ (Chi-squar) testing

Evaluation of the analytical specificity and analytical sensitivity of conventional PCR for the identification of *Corynebacterium pseudotuberculosis*

The specificity of PCR analysis for *Corynebacterium pseudotuberculosis* is conferred by the primers selected, which show 100% homology with the bacteria's DNA segments (16s RNA and PLD gene).The analytical specificity was evaluated using DNA from 2 different *Corynebacterium pseudotuberculosis* strains and DNA from other common bacteria in animal diseases, such as *Chlamydia sp., Mycobacterium avium paratuberculosis, Escherichia coli, Neospora caninum, Coxiella burnetti, Mycoplasma sp., Staphylococcus sp., Actinobacillus pleuropneumoniae, Pasteurella multocida,* among others, which were selected because of their pathogenic similarities (e.g., pyogenic lesion formation and toxic factor production) and of their hosts (small ruminants) (Figure 1).

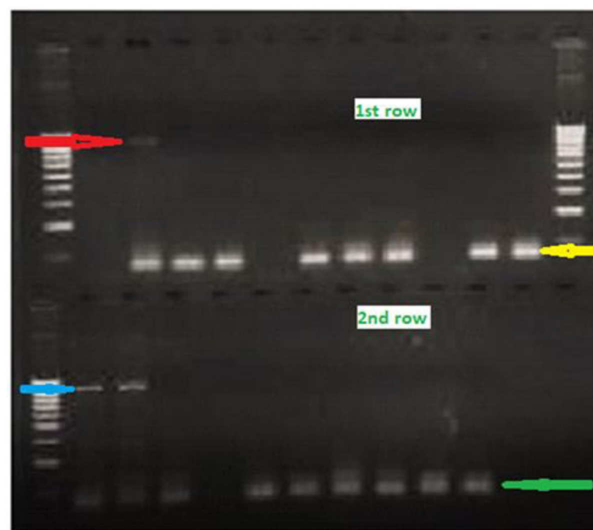

Figure 1: Electrophoresis analysis for the evaluation of the specificity of PCR for the detection of *Corynebacterium pseudotuberculosis*.
In the 1st row, from left to right, are the molecular weight marker, common PCR products for negative control and DNA of *Corynebacterium pseudotuberculosis* field strain (red arrow), and various other bacterial DNAs (orange arrow). In the 2nd row, from left to right, are the molecular weight marker, PCR products with acquired DNA from *Corynebacterium pseudotuberculosis*—DSMZ 20689 and *Corynebacterium pseudotuberculosis*—field strain (blue arrow), and PCR products with DNA from other bacteria (green arrow).

The PCR analysis results used to evaluate the specificity of PCR for *Corynebacterium pseudotuberculosis* allowed us to conclude that this test has high analytical specificity, as it was not possible to detect DNA from other bacteria with pathogenic characteristics and hosts analogous with the agent under study.

The sensitivity of PCR for the detection of *Corynebacterium pseudotuberculosis* was evaluated using successive dilutions of 25 ng of standard DNA-acquired strain DSMZ 20689 of *Corynebacterium pseudotuberculosis*-approximately equivalent to 2.5×10^7 CFU (colony - forming units) of *Corynebacterium pseudotuberculosis* (Figure 2).

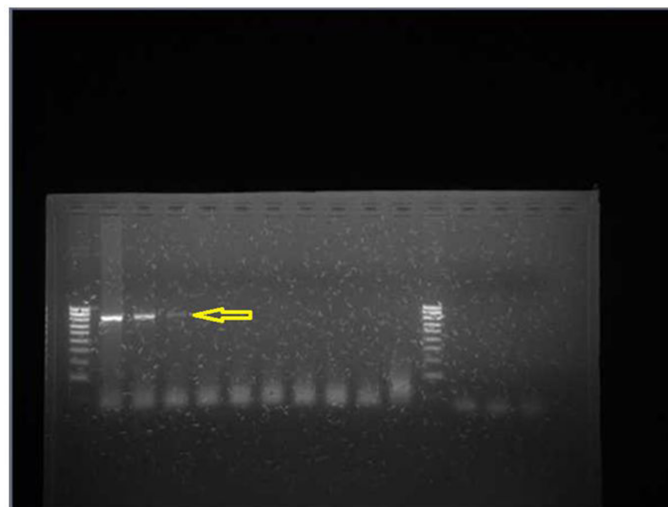

Figure 2: Electrophoresis analysis for evaluation the sensitivity of PCR for the detection of *Corynebacterium pseudotuberculosis* (from left to right, the molecular weight marker, the PCR product with 0.1 ng of standard DNA, and 1:10 dilutions in the subsequent wells, with a response observed at 0.001 ng of DNA / reaction, a dilution equivalent to 10^3 CFU of Corynebacterium pseudotuberculosis (yellow arrow))

PCR testing performed with serial dilutions (1:10) of 25 ng of standard DNA-strain DSMZ 20689 of *Corynebacterium pseudotuberculosis*-demonstrate that the PCR assay designed for the identification of Corynebacterium pseudotuberculosis has high analytical sensitivity, as it allows the identification of the agent in a DNA dilution at low as 0.001 ng, equivalent to 10^3 CFU of *Corynebacterium pseudotuberculosis.*

Results

Positive results in one or more of the four PCR products analysed are presented in Table 3. As seen in Table 3, from the 58 pyogenic samples analyzed, 10 were positive for at least one of the PCR product analyzed in this study. Of the 10 positive results, 3 were positive in PCR I, and 4 results were positive only in PCR III, which detected the 16S gene for *C. pseudotuberculosis* with genomic DNA diluted 1:10 (Figure 3 and Figure 4). Of the 58 clinical samples analyzed, 3 were positive for both PCR products I and II, with 16S and PLD gene segments for *C. pseudotuberculosis* detected at normal concentrations (Figure 4). As each sample originated from a different herd, we estimated an overall prevalence of 17, 24% for Caseous Lymphadenitis in affected herds throughout the Portuguese region of Alto Alentejo. Of the 10 samples with positive results, one was derived from goats (sample 84) and the remaining nine were obtained from sheep (Table 4).

Sample number	PCR I 16S Results (Normal DNA concentration)	PCR II PLD Results (Normal DNA concentration)	PCR III 16S Results (Diluted DNA 1:10)	PCR IV PLD Results (Diluted DNA 1:10)
4	NEGATIVE		**POSITIVE**	NEGATIVE
32	NEGATIVE		**POSITIVE**	NEGATIVE
52	**POSITIVE**	NEGATIVE	NEGATIVE	NEGATIVE
61	NEGATIVE		**POSITIVE**	NEGATIVE
62	NEGATIVE		**POSITIVE**	NEGATIVE
63	**POSITIVE**	NEGATIVE		NEGATIVE
66	**POSITIVE**	NEGATIVE		NEGATIVE
77	**POSITIVE**	**POSITIVE**		NEGATIVE
84	**POSITIVE**	**POSITIVE**		NEGATIVE
85	**POSITIVE**	**POSITIVE**		NEGATIVE

Table 3: Positive results obtained via PCR I, PCR II, PCR III and PCR IV with normal DNA concentrations (e.g.,1 μL and 2 μL) and diluted DNA concentrations (1:10).

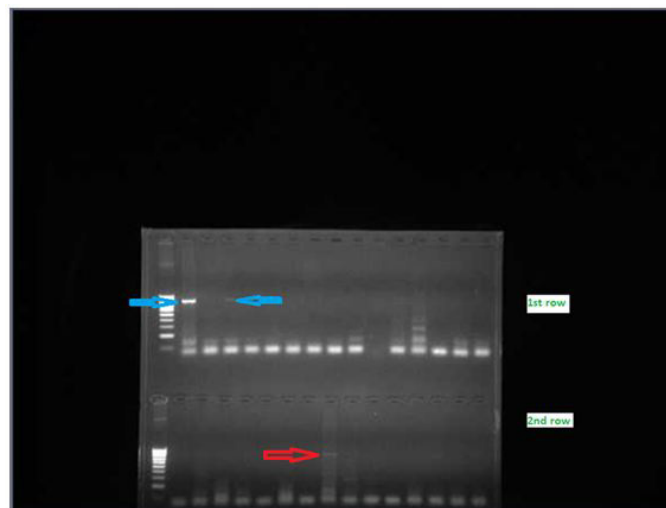

Figure 3: Electrophoresis analysis of the results of PCR I and PCR III (as seen in the 1st row, from left to right, the molecular weight marker (bands distributed from 1000 to 100 bp), followed by PCR products from samples 4 to 44-with positive samples identified in 4 and 32 (815bp) (blue arrows). In the 2nd row, from left to right, the molecular weight marker and PCR products from samples 45 to 59 sequentially, with sample 52 identified as positive (815 bp) (red arrow))

In the visceral form of the disease, the lung was the most-commonly affected organ, with pyogranulomatous lesions caused by *C. pseudotuberculosis* observed in 40% of the tested animals. The liver was the second-most affected organ (20%), and the disseminated form was identified only in one animal (Table 4).

Subcutaneous abscesses (cutaneous or external form of the disease) were observed in 30% of cases with identification of the etiologic agent of CL. In general, the visceral form was the most prevalent, with 70% of positive results in the identification of *C. pseudotuberculosis* in PCR samples obtained from internal lesions (Table 4).

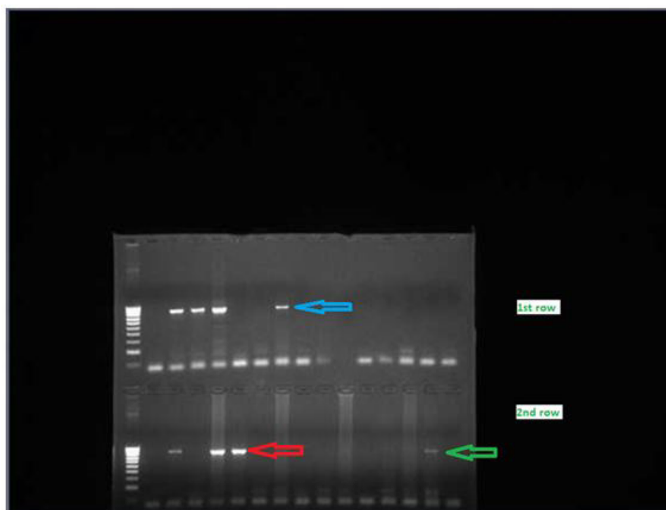

Figure 4: Electrophoresis analysis of the results of PCR III, PCR II and PCR I (in the 1st row, from left to right, the molecular weight marker, followed by PCR products from samples 60 to 68, followed by an empty well and PCR products from samples 69 to 73, with positive samples identified in samples 61, 62, 63 and 66 (815 bp) (blue arrow). In the 2nd row, from left to right, the molecular weight marker and PCR products from samples 76 to 85, followed by samples 51 II to 58 II, as well as positive and negative controls (green arrow), with samples 77, 84 and 85 identified as positive (815 bp) (red arrow))

	Organ/Affected area								
	Subcutaneous abscesses		**Liver**		**Lung**		**Disseminated form**		
Positive sample number	sheep	goats	sheep	goats	sheep	goats	sheep	goats	58 tested organs
	4				52		77		
		84	32		61				
	85		62		63				
					66				
Total number (%)	2 (20)	1 (10)	2 (20)	0	4 (40)	0	1 (10)	0	10 (17,24)
	30% superficial CL lesions		70% visceral CL lesions						

Table 4: Distribution of positive samples of *C. pseudotuberculosis* by organ/affected area and animal species

Statistical analysis of the observed results showed no statistically significant difference between species (sheep and goats) concerning positive PCR identification for *C. pseudotuberculosis* (P>0.05) (Figure 5).

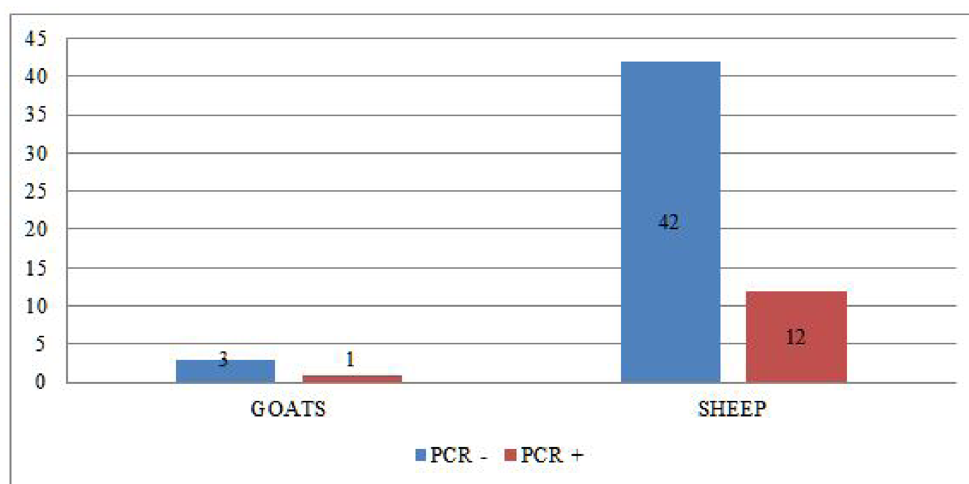

Figure 5: Distribution of positive and negative PCR samples for *C. pseudotuberculosis* by species (e.g., sheep and goats)

With regards to the location of the lesions, including both positive and negative results for *C. pseudotuberculosis* PCR analysis, the evaluated distributions did not prove to be statistically significant (P>0.05), although it was possible in Figure 6 to see that the lung was the organ most affected by pyogenic lesions (Figure 7).

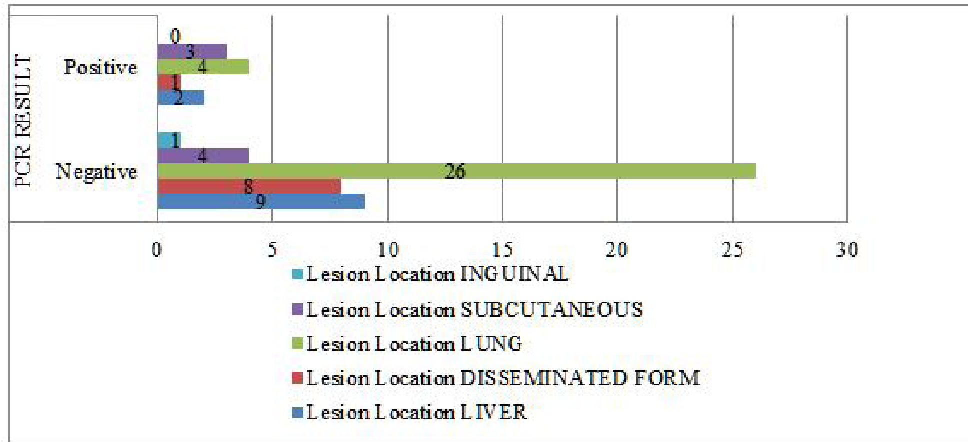

Figure 6: Lesions Location

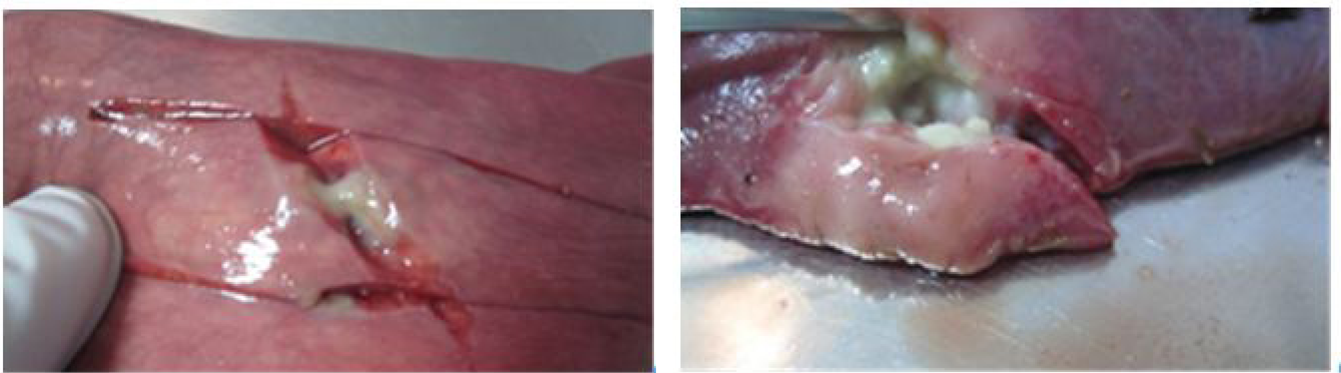

Figure 7: Pulmonary lesions in a sheep with identification of *C. pseudotuberculosis* (a) Cutting of the lung parenchyma with exit of thick purulent material; (b) Encapsulated pulmonary abscess

In contrast, when evaluating the distribution of pyogranulomatous lesions detected in sheep and goats, we found a statistically significant difference between the two species ($P<0.05$), with sheep having the higher frequency of characteristic CL lesions (Figure 8).

Figure 8: Distribution of pyogranulomatous lesions screened for *C. pseudotuberculosis* by PCR in sheep and goats

Discussion

The extraction process and purification of genomic DNA for the detection of *C. pseudotuberculosis* was hampered by the large pyogenic component of the pathological samples and a high concentration of cellular debris. The dilution of DNA samples allowed us to detect four positive samples in PCR III, in which we investigated the 16S gene for *C. pseudotuberculosis* with genomic DNA diluted 1:10 (Figure 4). Therefore, to detect the maximal number of positive animals in the diagnosis of CL, it

is appropriate to perform PCR analysis by searching for more than one genomic DNA fragment (i.e., 16s RNA and PLD) while diluting the DNA samples.

Molecular diagnosis is not a routine analysis technique in screening of caseous lymphadenitis, mainly because of the high cost of PCR testing. However, compared to other diagnostic methods available for this disease, PCR has proven to be a sensitive and specific test that enables the identification of an etiologic agent from a wide variety of pathological samples.

The definitive diagnosis of CL is primarily based on culture and identification of *C. pseudotuberculosis*, which can be isolated from any kind of lesions. However, the number of viable bacteria in a chronic abscess may be low, and sterile lesions can occasionally be found [11]. A study in Canada that focused on CL identified *Corynebacterium pseudotuberculosis* (via bacterial isolation) in 37 of 54 animals with abscesses [28]. In the 17 negative animals, the authors isolated *Trueperella (Arcanobacterium) pyogenes* in 6 cases, *Pasteurella multocida* in one case and isolated no bacteria in 10 cases, consistent with the possibility of "sterile" abscesses or abscesses with very low microbiological levels.

The present study allowed us to evaluate the analytical specificity and sensitivity of conventional PCR testing for *C. pseudotuberculosis*. It was not possible to obtain a relative specificity or sensitivity value because no comparative tests were performed to check for false positives or false negatives; nevertheless, high analytical specificity and high analytical sensitivity of PCR testing for *Corynebacterium pseudotuberculosis* were identified.

From the 335 animals observed in the slaughterhouse, 58 (17, 31%) were presumptively diagnosed with CL. *C. pseudotuberculosis* was identified in 17, 24% (10/58) of these animals. The overall CL prevalence in affected flocks of small ruminants (17, 31% in macroscopic observation of lesions and 17, 24% in agent identification by PCR) confirmed in this investigation was lower than that obtained in previous studies. This prevalence may have resulted from the chronic and purulent nature of the study samples, which may correspond to CL lesions that lack the etiologic agent or its DNA, thus leading to an underestimation of the true prevalence of CL. In a recent study in Korea in which 466 Korean native goats were tested, 34 (7.3%) were grossly diagnosed with CL. *C. pseudotuberculosis* was isolated from 24 of these goats (70.6% of goats with superficial CL lesions), with infection confirmed by PCR [29]. In the province of Tanta, Egypt, a study performed in a slaughterhouse identified 259 animals severely affected with CL, among 962 small ruminants slaughtered, representing a prevalence of 26.92% [30]. *Corynebacterium pseudotuberculosis* was isolated (via bacteriological analysis) in 241 cases (25.05% of the total).

The majority of pathological samples were obtained from sheep, which is consistent with data indicating that CL is more prevalent in sheep than in goats. The increased prevalence of CL may be because, in the study area, there is a predominance of ovine species (approximately 250 000 sheep and 87 000 goats) [31-33]. However, one of the positive PCR identification results was found in a goat sample (10%). Several authors have suggested that goats are more commonly affected by external abscesses, while the visceral form of CL may be more common in sheep [17,34].

Regarding lesion locations, the lung was the most-commonly affected organ in the visceral form of the disease (40%), as previously described in the literature [17,34,35]. Visceral disease presentation was the most frequent, with the identification of *C. pseudotuberculosis* in a greater percentage of visceral lesions (70%) compared with the skin or superficial forms (30% of subcutaneous abscesses). These data confirm previous study results, demonstrating that the presence of external abscesses represents only the tip of the iceberg, and underscoring that the problem of CL in the visceral form is more prevalent than commonly considered [17,34,35]. Small ruminants affected by the visceral form of the disease rarely show abscesses in external lymph nodes, which emphasizes the importance of accurately detecting animals affected by the visceral form [17].

This is the first prevalence study of caseous lymphadenitis in small ruminants carried out in Portugal. The only work previously published Boinas F *et al*. used a very small number of animals and flocks [36].

Approximately half of the national herds of small ruminants (46%) reside in the Alentejo region, and the annual production of lamb meat and certified sheep cheese has its largest representation in Alto Alentejo [31-33,37]. We therefore conclude that the presence of a disease with such insidious characteristics and chronic nature as CL at such a high prevalence require the concern of public health authorities and research centres of animal health. Future studies should attempt to estimate its morbidity and mortality rate and its overall economic impact for the country and the region [38,39].

The performance of part of the study in a slaughterhouse allowed the real evaluation of CL on actual farms, including the detection of subclinical animals.

This study faced certain limitations, including the inability of identifying other etiologic agents in pyogenic lesions, which would have increased overall knowledge for differential diagnoses of CL and, non-performance of bacterial culture (gold standard technique for detection of *C. pseudotuberculosis*) for comparison with PCR results.

PCR proved to be an excellent test for definitive and differential diagnosis of CL, although it is not the most appropriate for prevalence studies due to its relative expense compared to microbiological culture. Furthermore, PCR is not likely to become the technique of choice in control and eradication programmes for CL since it does not allow for *in vivo* detection of subclinical

carriers, the main cause of disease spread within a flock.

Additional studies concerning the true prevalence and economic impact of CL should be conducted to more fully assess the importance of this disease in Portugal and in the Alentejo.

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None of the authors declare any competing interests regarding the manuscript.

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