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Corynebacterium pseudotuberculosis: Immunological Responses in Animal Models and Zoonotic Potential

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Abstract

Corynebacterium pseudotuberculosis is a member of the Corynebacterium, Mycobacterium and Nocardia (CMN) group that comprises species of medical, veterinary and biotechnological interest. This pathogen mainly affects small ruminants, causing caseous lymphadenitis (CLA), but it also infects bovines, equines, pigs, deer, camels and humans, showing its zoonotic relevance. Phospholipase D (PLD) and the toxic lipid cell wall are the two most well-studied virulence factors of this bacterium. They are responsible, in part, for the establishment of disease in the host. Current knowledge on the immunity induced by *C. pseudotuberculosis* indicates that the resistance to infection is a complex process involving components of both the non-specific and specific host responses, in which humoral and cellular immune responses are both operative. Despite this knowledge and the importance of the disease, a satisfactory vaccine model for sheep and goats has not been developed. Moreover, a control program that includes an efficient diagnostic method in addition to vaccination is crucial for avoiding the spread of bacteria inside flocks. Further, because of its zoonotic potential, *C. pseudotuberculosis* infection of animals can contaminate meat and milk, putting consumers at risk. The ability of *C. pseudotuberculosis* to infect both animals and humans makes studies on prevention and diagnosis of this pathogen important.

Introduction

The genus *Corynebacterium* is classified into the suborder Corynebacterineae (Actinobacteria: Actinomycetales), which includes the families Corynebacteriaceae, Mycobacteriaceae and Nocardiaceae (http://www.ncbi.nlm.nih.gov/Taxonomy), commonly designated as the CMN group. Some characteristics common to this group include the organization of the cell wall, which is composed mainly of peptidoglycan, arabinogalactan and mycolic acids, as well as the high guanine-cytosine (G + C) content of the genome (47-74%) [1].

The CMN group comprises species of significant medical, veterinary and biotechnological interest, and thus has an important socioeconomic role. For example, the CMN group includes the species *Mycobacterium tuberculosis* and *M. leprae*, the etiologic agents of tuberculosis and leprosy, respectively. Within the genus *Corynebacterium*, there has been a significant expansion in the number of described species in the last decade due to increased concern regarding their potential pathogenic significance. The species that are of major clinical relevance are *Corynebacterium diphtheriae*, *C. jeikeium* and *C. pseudotuberculosis*, which are the causative agents of diphtheria, nosocomial infections in humans and caseous lymphadenitis (CLA) in goats and sheep, respectively [1-3]. *C. glutamicum* is used in the production of amino acids such as L-aspartate and L-lysine [4], and *C. ulcerans* causes pathologies in both humans and animals [5].

Most studies mainly report the incidence of *C. pseudotuberculosis* infection in small ruminants. Affected animals have characteristic abscesses in the lymph nodes, mainly in the parotid or retropharyngeal and/or viscera [6]. However, some infected sheep may have only internal abscesses, which are often present in the lungs or mediastinal lymph nodes, and show no major outward signs of infection [7]. This

pathogen has a broad spectrum of hosts and causes clinical disease in cattle, horses, pigs, deer, camels and laboratory animals [8]. According to the World Animal Health Organization, among the 201 countries that described their sanitary conditions, 64 reported animals with CLA within their borders in 2009 [9]. Although human cases of infection are relatively rare, some case reports note the potential risks of infection of veterinarians and farm practitioners [10]. In this review, we present the general characteristics and virulence factors of *C. pseudotuberculosis* related to its immunopathogenesis, the response of the immune system, aspects associated with resistance to infection in animal models and diagnostics. We also discuss the zoonotic potential of the bacteria, presenting an update on human infections by *C. pseudotuberculosis*. Finally, we suggest guidelines for future research.

General Characteristics of *Corynebacterium pseudotuberculosis*

Corynebacterium pseudotuberculosis is the etiological agent of CLA, which mainly affects populations of small ruminants throughout the world and generates significant economic losses [11]. This bacterium was first described in 1888 by a French veterinarian, Edmound Nocard,

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in a case of lymphangitis in cattle. In 1891, Hugo von Preisz isolated a similar bacterium in a renal abscess in sheep. Hence, the pathogen was named "Preisz-Nocard" bacillus, the name with which it was linked for decades thereafter. In Australia, which is currently the country with the second largest sheep flock in the world, CLA was firstly described in 1934 by Churchward [8,12,13].

The current nomenclature was adopted in 1948 in the sixth edition of Bergey's Manual; however, the designation *C. ovis* was used synonymously. *C. pseudotuberculosis* is characterized as a Gram-positive facultative intracellular pathogen that displays pleomorphic forms, ranging from coccoid to filamentous rods and measures approximately 0.5-0.6 μm in width and 1.0-3.0 μm in length. These bacteria do not have a capsule or flagella and they do not sporulate; however, fimbriae are present. The cell-wall peptidoglycan appears to be based on meso-diaminopimelic acid (meso-DAP), and the sugars arabinose and galactose are present in large quantities. Short chains of mycolic acid have also been found [14-16].

C. pseudotuberculosis is a mesophilic facultative anaerobic organism, and its optimum growth conditions are at 37ºC with pH values ranging between 7.0 and 7.2 [12,17]. However, according to Batey [18], *C. pseudotuberculosis* can grow well in a pH range of 7.0 to 8.0. *C. pseudotuberculosis* is a demanding organism from a nutritional standpoint, growing well on enriched media such as blood agar, brain heart infusion (BHI) agar or broth or enriched medium with animal serum. Its cultivation improves when BHI is complemented with yeast extract, tryptone or lactalbumin [19]. Its growth in a solid culture medium is initially sparse on an agar surface and then becomes organized in clumps or in palisades, taking on a cream to yellowish coloration. Colonies are dry, opaque and concentrically ringed, and after several days of incubation, colonies can reach 3 mm in diameter [20]. When grown in blood agar, it is possible to detect beta-hemolysis after a period of 48 to 72 hours of incubation [19,20]. Growth in liquid medium occurs as a biofilm on the surface, without medium clouding. This film is dismantled by agitation, forming flakes that precipitate [17,21]. The film formation is attributed to surface lipids, and the amount of these lipids in the cell membrane is directly correlated with the thickness of the biofilm and the virulence of the bacterial strain [22]. Some authors have reported better growth in an atmosphere of 5% CO_2 [12,21]. Recently, a chemically defined medium (CDM) for *C. pseudotuberculosis* cultivation composed of dibasic phosphate, vitamins and amino acids was developed. This medium is free of macromolecules in its composition, allowing researchers to obtain the complex antigenic solution composed only of secreted/excreted bacterial proteins, which would aid studies on this bacterium [23]. This CDM has been recently used with success to obtain pure secreted antigens for an exoproteome analysis of *C. pseudotuberculosis* [24].

Biochemically, *C. pseudotuberculosis* is characterized by the production of catalase, phospholipase D, and urease and by the fermentation of carbohydrates such as maltose, mannose, glucose, and galactose (the latter is used only occasionally) [17]. It does not perform lactose fermentation or gas production [21,25-27], has no proteolytic activity, does not have the ability to hydrolyze gelatin or digest casein and is oxidase negative [17,20]. It has been suggested that there are two biotypes of these bacteria based on the production of nitrate reductase, demonstrated by analysis with restriction enzymes. The reduction of nitrate to nitrite characterizes strains that infect mainly horses (biovar *equi*) and present sensitivity to streptomycin, while the strains that infect goats and sheep (biovar *ovis*) are mainly nitrate negative and resistant to streptomycin [27,28]. Cattle are infected by both reducing and non-reducing nitrate strains [25,26].

Antigens and Virulence Factors

Compared to other pathogenic bacteria, few studies have investigated the virulence determinants of *C. pseudotuberculosis*. However, an understanding of the molecular mechanisms and the genetic basis of virulence in *C. pseudotuberculosis* has rapidly improved. Two virulence determinants of *C. pseudotuberculosis* have been well characterized: the exotoxin PLD and the toxic lipid cell wall. PLD hydrolyzes phosphatidylcholine and sphingomyelin from mammalian cell membranes, resulting in the formation of choline and ceramide phosphate [29-31]. Biologically, this exotoxin works as a permeability factor that promotes the dissemination of the pathogen from the initial site of infection to all the body tissues of the host. Additionally, PLD causes the dermonecrosis of endothelial cells, which contributes to the passage of *C. pseudotuberculosis* from the dermis to small blood vessels, thereby gaining access to lymphatic vessels [32,33]. Further, PLD is considered a cytotoxic exotoxin for white blood cells, as it promotes the destruction of goat macrophages during experimental infection [34].

Studies using specific anti-PLD antibodies have demonstrated that this exotoxin is essential to the spread of *C. pseudotuberculosis*, as the use of this antibody prevents its spread. In addition, the vaccination of goats with inactivated exotoxin is able to prevent the spread of the bacteria after experimental challenge [35]. The role of PLD as a virulence determinant of *C. pseudotuberculosis* was demonstrated by the generation of *pld*-mutants. The mutant was unable to spread but did induce an immune response. However, these results suggest that the use of this mutant as a live vaccine model for CLA would be insufficient [36]. The spread of bacteria by the action of PLD can be explained by its induction of dermonecrosis, which causes direct damage to endothelial cells. During hydrolysis, PLD is able to work synergistically with cholesterol oxidase and phospholipase C, which are both, produced by *Rhodococcus equi*, and causes the β-hemolysis of sheep erythrocytes when cultured in blood agar. The phosphate ceramide generated, in turn, is hydrolyzed by phospholipase C of *R. equi*, producing ceramide [14,30]. In addition to the degradation of sphingomyelin to ceramide, PLD is also able to activate the complement system through the alternative pathway, although the exact mechanism is not well understood [37].

A 40-kDa protein from *C. pseudotuberculosis* that was previously identified as a protective antigen against ovine CLA [38] was further investigated. Molecular and biochemical characterization revealed that this protein is a 379 amino acid protein encoded by an open reading frame of 1,137 bp. No significant homology with previously published DNA or amino acid sequences in the databases was found, suggesting that this is a novel protein. Thus, the recombinant 40-kDa protein was over-expressed in *E. coli* for the biochemical analysis of the native and the recombinant 40-kDa proteins. The protein was found to be a serine protease with proteolytic activity and was designated corynebacterial protease 40 (CP40) [39].

Other antigens from both the secreted and somatic fractions were identified by an immunoblotting analysis. One study used the serum of naturally infected sheep for immunoblotting to identify antigens from sonicated *C. pseudotuberculosis* and found eleven antigens with the following molecular weights: 20 kDa, 22.4 kDa, 31.6 kDa, 35.5 kDa, 36.3 kDa, 39.8 kDa, 45.7 kDa, 56.2 kDa, 63.1 kDa, 79.4 kDa and 100

kDa. When the analyses were performed with secreted antigens from the culture supernatant, antigens with the following molecular weights were found: 20 kDa, 25.1 kDa, 31.6 kDa, 39.8 kDa and 63.1 kDa [40]. A previous immunoblotting analysis with sera from experimentally infected goats on secreted antigens from bacterial culture supernatants revealed antigens with the following molecular weights: 16, 20, 27, 30, 36, 40, 43, 58, 64, 68 and 125 kDa [41].

The introduction of more advanced technologies such as mass spectrometry (MS) has brought new perspectives to the study of bacterial secreted/excreted proteins. A comparative exoproteome analysis of *C. pseudotuberculosis* was recently performed using a high-throughput proteomic strategy based on liquid chromatography-mass spectrometry (LC-MS). This study investigated the proteins that are exported from the bacteria, which might represent key components of the host-pathogen interplay. Ninety-three different extracellular proteins of *C. pseudotuberculosis* were identified with high confidence using this strategy; 44 proteins were commonly identified in two different strains that were isolated from distinct hosts, thus composing the core of the *C. pseudotuberculosis* exoproteome. This study generated a catalog of exoproteins, in which novel targets for future work on *C. pseudotuberculosis* molecular determinants of virulence can be identified [24].

As *C. pseudotuberculosis* is classified in the Actinomycetales order, it shares some common characteristics with this group. *C. pseudotuberculosis* has a significant amount of lipids in its cell wall called corynomycolic acids, and they are similar to the mycolic acids in *M. tuberculosis* cell walls. These surface lipids have long been described as important factors for the pathogenesis of the disease, as virulent strains have more lipids than attenuated strains, and there is a direct relationship between the percentage of surface lipids and chronic abscesses [21]. This lipid layer protects against proteolytic enzymes present in phagolysosomes, allowing the microorganism to adhere, thereby promoting local cytotoxicity. The toxicity of the extracted lipid material was demonstrated by the induction of hemorrhagic necrosis after intradermal injection into guinea pigs [22,42,43].

Recently, four genes within an operon that is involved in iron acquisition, designated *fag* A, B, C and D, were shown to have an important role in the virulence of *C. pseudotuberculosis* [44]. Recent advances in the understanding of pathogenicity pathways used by *C. pseudotuberculosis* in the infection process have been achieved through complete genome sequencing [45]. A *C. pseudotuberculosis* strain (FRC41) isolated from a 12-year-old girl with necrotizing lymphadenitis was completely sequenced and assembled, yielding the identification of specific gene sets associated with a variety of metabolic and pathogenic bacterial functions. Two gene clusters encoding proteins involved in the sortase-mediated polymerization of adhesive pili were found, and these proteins likely mediate the adherence to host tissue, thereby facilitating additional ligand-receptor interactions and the delivery of virulence factors. In addition, the prominent virulence factors PLD and CP40 were found in the genome of this human isolate. The genome also revealed additional serine proteases, neuraminidase H, nitric oxide reductase, an invasion-associated protein, and acyl-CoA carboxylase subunits involved in mycolic acid biosynthesis as potential virulence factors [46].

The genetic characterization of two strains of *C. pseudotuberculosis* from goat (Cp1002) and sheep (CpC231) using a predicted genome analysis contributed new information on genome evolution and lateral acquisition of virulence functions. *C. pseudotuberculosis* was compared with other *Corynebacterium* species, revealing that this pathogenic species has lost numerous genes, resulting in one of the smallest genomes in the genus. Other differences found were a lower GC content (approximately 52%) as well as a reduced gene repertoire. These characteristics suggest adaptations that give the bacteria its pathogenesis. In addition, seven putative pathogenicity islands were found in its genome that contain several classical virulence factors, including genes for fimbrial subunits, adhesion factors, iron uptake and secreted toxins [9].

Immunopathogenesis of CLA

The progression of CLA in sheep and goats starts as primary wound infection, with lymphatic and hematogenous dissemination, followed by secondary infection of the lymph nodes and various visceral organs. This is followed by the elimination or containment of infection, the latter presenting as characteristic caseous lesions. The steps of infection have been separated into the following phases: an initial phase (day 1–4 p.i.), characterized by the recruitment of neutrophils to the inoculation site and the draining of the lymph nodes; an amplification phase (day 5–10 p.i.), characterized by the development of pyogranuloma; and a stabilization phase, characterized by the maturation and persistence of the pyogranuloma [47]. Bacterial factors, including PLD and cytotoxic lipids, contribute to pathogenesis at a local level but have little effect on the systemic disease. After *C. pseudotuberculosis* is captured by phagocytic cells such as neutrophils and macrophages, the phagosome fuses with the lysosome, forming the phagolysosome [28]. However, *C. pseudotuberculosis* is a facultative intracellular pathogen that is capable of surviving within macrophages for more than 48 hours. During that time, bacteria are released as a result of a process that leads to phagocyte death, although this property varies among different strains. The specific mechanisms of cell death caused by *C. pseudotuberculosis* are still unclear, as it does not induce the autophagy or apoptosis of macrophages. This has been demonstrated in murine macrophage cell lines, as evidenced by stable levels of microtubule-associated protein I light chain 3 (MAP-I LC3) activity and caspase-3 activity and an absence of nuclear fragmentation in infected macrophages [48]. The bacteria survive within macrophages because some macrophages cannot produce nitric oxide in response to *C. pseudotuberculosis in vivo*, which results in ineffective clearing of the organism [49]. These effects might be associated with the outer lipid layer in the cell wall of *C. pseudotuberculosis* and other antigenic components that attenuate the production of nitric oxide by macrophages.

As a result of the uncontrolled bacterial growth within macrophages, the host attempts to restrain and limit the infection through the formation of pyogranulomas, which are characterized by the encapsulation of the *C. pseudotuberculosis* infected cells. The formation of pyogranulomas is dependent on adaptive immunity, which is a complex process in the case of infection by *C. pseudotuberculosis* that involves both humoral and cell-mediated immunity [28,50]. Immunohistochemical studies on the cellular composition of the pulmonary lesions in sheep infected by *C. pseudotuberculosis* have revealed a predominance of large macrophages that express major histocompatibility complex (MHC) class II molecules on their surfaces in the abscess walls and surrounding lung parenchyma. T lymphocytes were prominent in the same areas within the naturally occurring lesions, with a CD4+ T cell to CD8+ T cell ratio of 3.5:1. B lymphocytes and granulocytes comprised a minor portion of the infiltrating cells. These data revealed the participation of macrophages and MHC class II-restricted T lymphocytes in the pathogenesis of CLA [51].

Another immunohistochemical study revealed more features of the pyogranuloma cellular makeup. Lymphocytes localized to the inside of the collagen capsule were found to be in close contact with necrotic tissue and were organized into three layers. The innermost layer, which is immediately adjacent to the central necrotic tissue, consists of a narrow band of MHC class II-macrophages. CD4^{+}, CD8^{+} and gamma delta T cells were unevenly distributed throughout the lymphoid layer, tending to be more numerous immediately peripheral to the macrophage layer. The intracapsular lymphoid tissue contained a high proportion of CD8^{+} lymphocytes (CD4:CD8, 1.5:1) and gamma delta lymphocytes (CD4:CD8: gamma delta, 1:0.7:0.8). However, in contrast to the previous immunohistochemical study, CD8^{+} and gamma delta^{+} T cells were found to be more abundant than CD4^{+} T cells [52].

Recent studies have clarified the cellular makeup of pyogranulomas. In an experimental pyogranuloma induced in lambs infected with *C. pseudotuberculosis*, three different monoclonal antibodies were used to characterize the presence of macrophages *in situ* and demonstrated that macrophages are the predominant cells in pyogranulomas [53]. Further immunohistochemical experiments on the cellular composition of 46 experimentally induced pyogranulomas in sheep also demonstrated that lesions localized to inoculation sites or draining lymph nodes consisted of macrophage and lymphocyte layers distributed around the necrotic center, surrounded by a fibrous capsule. However, the most significant observation from this work was that in immature lesions, CD4^{+} T cells were most predominant, whereas in mature lesions, the proportions of CD8^{+} T lymphocytes and gamma/delta receptor-expressing cells increased. In addition, this study found that many pyogranuloma cells expressed the interleukin-2 receptor, and a large individual variability in the proportions of macrophage and T cell subsets was observed in lesions at the same maturation point, with particular discrepancies in the number of epithelioid macrophages. This heterogeneity suggests that there are different cellular patterns based on the persistence or elimination of bacteria by the host [54].

A recent study aiming to evaluate the outcomes in mice after inoculation with four equine-origin *C. pseudotuberculosis* strains (slow and rapid-growing strains) demonstrated that the tropism for the liver, spleen, lungs, and mesenteric lymph nodes is distinct for each strain. Specifically for the liver, the histological lesions associated with rapidly growing strains included focally extensive unencapsulated areas of acute, massive coagulative necrosis of hepatocytes. These areas had intralesional colonies of bacteria and variable portal hepatitis characterized by the accumulation of mononuclear and polymorphonuclear inflammatory cells. In contrast, the livers from mice inoculated with slow-growing strains had multiple discrete, randomly distributed foci of hepatocellular necrosis and neutrophilic hepatitis that were considerably less severe than the lesions in the mice inoculated with the rapidly growing strains [55].

In addition to the formation of pyogranulomas, infection with *C. pseudotuberculosis* leads to the formation of subcutaneously located abscesses that do not originate from primary lymph node lesions. However, some abscesses can be closely association with lymph nodes, and as a result of the tissue destruction associated with abscesses and the expanding nature of these lesions, micro-abscesses that are composed of multiple caseous lesions of 0.5–1.0 cm in diameter can occur. Microscopically, these encapsulated abscesses contain partially calcified pus arranged in a concentric lamellar structure [56].

Resistance to *C. pseudotuberculosis*

Resistance to infection by *C. pseudotuberculosis* is a complex process involving components of both the non-specific and specific host response, in which both the humoral and cellular immune responses are operative [28,57,58]. The importance of humoral mechanisms has been demonstrated by assessing numerous commercial and experimental vaccine trials, which include preparations based on inactivated cell-culture supernatant or toxoids [59,60], bacterial cell-wall fractions [61,62], attenuated and killed bacteria [63,64], exotoxins and their subunits [34,65] or genetically modified pathogens [66,67]. Advances regarding immunoprophylaxis have been made, and the use of these preparations reduces the number and size of granulomas in challenged animals. However, to adequately control infection, the activation of macrophages needs to be improved; indeed, many researchers are now focusing on the gamma IFN (IFN-γ) response.

Early studies in mice evaluated the ability of levamisole to promote nonspecific immunity to *C. pseudotuberculosis* infection. The enhanced nonspecific resistance was demonstrated with a quantitative reduction in immunoglobulin levels; the levels of IgG2 and IgA were lower in mice pretreated with levamisole. This result suggested that cell-mediated immunity might play a more important role than humoral immunity in resistance to *C. pseudotuberculosis* infection [68]. The role of IFN-γ was first demonstrated in experimental infections of mice deficient for the IFN-γ receptor with attenuated *C. pseudotuberculosis* mutants. The study found that the production of IFN-γ and the presence of its receptors were directly associated with the control of primary infection in mice [63]. Another important cytokine that controls primarily infections by *C. pseudotuberculosis* is tumor necrosis factor alpha (TNF-α) [63,69,70]. The administration of anti-TNF-α and anti-IFN-γ-monoclonal antibodies (mAbs) increased bacterial proliferation in infected mouse organs, leading to the death of the animals. Further, the injection of anti-CD4 or anti-CD8 mAbs also resulted in significantly increased mortality and a marked suppression of IFN-γ production but had no effect on TNF-α production. Therefore, endogenous TNF-α and IFN-γ may have addictive effects on the anti-corynebacterial defense in the early stage of infection and could be critical for the generation of resistance [69].

The same group also investigated the role of these cytokines during secondary *C. pseudotuberculosis* infection in mice. They found that both TNF-α and IFN-γ are required for survival and the development of protective immunity. During secondary infection, the mice recovered 50% faster, suggesting the importance of these cytokines in activating macrophages. In this trial, the administration of anti-CD4 mAb alone or anti-CD4 plus anti-CD8 mAbs increased mortality, bacterial proliferation and reduced the production of these cytokines, while treatment with anti-CD8 mAb alone showed no effect on either the resistance to infection or cytokine production. In conclusion, these data suggest that CD4^{+} T cells, likely Th1 T cells, play an important role in immunity against secondary *C. pseudotuberculosis* infection [71].

Another study on the innate immune response during the early course of infection revealed the participation of the complement system in the defense against *C. pseudotuberculosis*. Experiments demonstrated that the type 3 complement receptor (CR3) plays a key role in inflammatory cell recruitment during the course of infection. Indeed, the treatment of mice with an anti-CR3 mAb resulted in the unrestricted proliferation of bacteria in the spleen and livers and dramatically increased the mortality of the infected mice within

3 days of infection. A histological examination also revealed that mononuclear phagocytes did not migrate to the sites of bacterial multiplication, as evidenced by the inhibition of inflammatory cell migration and large numbers of bacteria in their organs. These results revealed the importance of CR3 in the resistance against primary as well as secondary *C. pseudotuberculosis* infection in mice [72].

Although the previous results highlight the importance of T cell-mediated immunity against the infection, some evidence has also indicated that the production of antitoxin may protect the host during secondary exposures to the pathogen [14,57]. In mice previously immunized with washed bacterial suspensions, the antitoxin was unable to prevent the formation of pus after inoculation, despite the fact that the antitoxin was able to prevent the spread of infection from the site of inoculation to internal organs [57]. These results were later reproduced in several experiments demonstrating that anti-PLD antibodies present in host blood before infection exert a protective effect by neutralizing the permeability induced by PLD, thereby hindering the dissemination of *C. pseudotuberculosis* to draining lymph nodes. Further, the antibodies reduced secondary spread and prevented lesion development [73].

As CLA affects sheep and goats, a vast number of studies have been performed in these species since the 1980s [28,50-54,62,74]. A groundbreaking study was conducted in sheep inoculated with attenuated and wild-type strains of *C. pseudotuberculosis*; the authors assessed the expression of cytokine mRNA during infection. This study revealed that at the site of experimental inoculation, the levels of the inflammatory cytokines TNF-α and interleukin-1β (IL-1β) were increased, and the expression of interleukin-4 (IL-4) was decreased. Further, the cytokines IL-2 and IL-4 were up-regulated. The highest expression of TNF-α, IL-2 and IFN-γ occurred at the seventh day post-inoculation, and the highest levels of IL-1β and IL-6 mRNA were detected 28 days after inoculation. The levels of mRNA for IL-1β, IL-6, IL-8, TNF-α and MCP-1 (monocyte chemoattractant protein 1) were elevated in animals with granulomas in the draining lymph nodes. These results suggested that the development of granulomas could be a consequence of the presence of Th1 and Th2 cells as well as the elevated production of cytokines such as IFN-γ, IL-2, IL-4, TNF-α and MCP-1. These data suggest that granulomas constitute an important factor for limiting *C. pseudotuberculosis* infection [47].

Another study assessed the kinetics of IgG and IFN-γ production in *Corynebacterium pseudotuberculosis* primary infection in goats. The authors demonstrated that the patterns of IgG production varied between animals, but the maximum titers were detected between days 11 and 21 post infection (PI). The levels further declined until 140 days PI. Serological positivity was detected from days 6 to 11 PI, but not all individuals remained positive throughout the 20 weeks of follow-up, indicating that humoral immunity is not long lasting. The same work demonstrated the existence of two patterns of the IFN-γ response, one considered high and another considered medium/low. In general, IFN-γ production was observed as a short-lived primary response on day 5 PI for the animals of both groups and a strong secondary response starting on day 16 and declining from days 42 to 56 after infection in the high response group. The "IFN-γ low producer" group exhibited only a short-lived peak on day 5 after infection and thereafter displayed no further significant antigen-specific cell-mediated stimulation [50]. This wide variation in the profiles of IFN-γ production was recently confirmed in a large study of sheep and goats. These differences have necessitated the use of assays with low sensitivity for measuring IFN-γ produced by peripheral leukocytes after stimulus with *C. pseudotuberculosis*; nonetheless, the production of IFN-γ remains a highly specific indicator of CLA [75].

The role of the innate immune system in *C. pseudotuberculosis* infection is currently under investigation, including the response pro-inflammatory cytokines and the acute phase protein. Previous studies in sheep have demonstrated an acute phase response during the initial phase of infection, with a significant increase in the concentrations of haptoglobin approximately 5 days PI [76]. A recent study confirmed the acute phase reaction of haptoglobin in CLA but also revealed that serum amyloid A (SAA) and α1 acid glycoprotein (AGP) were increased in an experimental model of CLA in sheep. The study also revealed the continued production of AGP during the transition to the chronic phase of the disease. These data suggest that AGP may be a valuable quantitative marker of the level of pathogenesis in experimental studies of CLA as well as a useful biomarker of natural infection and of chronic conditions in sheep [74]. Another recent study demonstrated that sheep that did not develop clinical signs of CLA presented significantly elevated levels of haptoglobin during the acute phase of the disease compared to sheep that developed superficial abscesses. Although the exact participation of this protein in the defense against infection by *C. pseudotuberculosis* requires further investigation, this suggests that innate immune mechanisms contribute to the resolution of infection or resistance to the development of CLA pyogranulomas. Further, haptoglobin has been suggested as a promising candidate marker for the clinical progression of *C. pseudotuberculosis* infection in sheep [77].

Vaccine Models Tested for CLA

Studies have shown that primary infection with viable bacteria induces strong protection against subsequent exposures. Indeed, ewes with primary infection did not develop lesions as a result of further exposure, whereas immune-naïve ewes developed numerous pyogranulomas at different sites. However, ewes with primary infection remained carriers of the bacterium as a result of primary inoculation [78,79]. A study conducted in alpacas confirmed this observation, showing that primary infection with a low dose of 1.1×10^3 CFU protected animals against a significantly higher dose of 9×10^8 CFU of *C. pseudotuberculosis*. The primary infected alpacas had a febrile response and abscesses at the inoculation site and regional lymph nodes; however, after the challenge, the primary infected animals showed no superficial lesions, in contrast to the immune-naïve alpacas that developed severe disease characterized by fever and abscesses in regional lymph nodes. In addition, primary infected alpacas had a robust antibody response against a *C. pseudotuberculosis* cell wall antigen [61]. These studies have encouraged scientists to create a vaccine model based on the use of killed bacterial cells, called bacterin. Killed *C. pseudotuberculosis* cells can theoretically mimic the primary infection, promoting immunoprotection without causing disease. A summary of data from studies published in the last 40 years on vaccine models is presented in Table 1, including the country of origin of the research, the animal model, the composition of the immunogens, the type of adjuvants used and the route of administration of the preparations. Killed bacteria have been used by many scientists as potential vaccines [59,62,80-84]. The degrees of protection have varied among these studies, but a common feature is that the protective effects were only partial. The immunogens did not completely prevent the disease, but the clinical course was milder, with significantly lower numbers of granulomas in immunized animals compared to unvaccinated control animals.

Another vaccine model uses the secreted exotoxins of *C.*

Reference	Year of publication	Country of origin	Animal model	Composition of the immunogens	Type of adjuvants	Route of administration
[81]	1972	Australia	Sheep	Bacterins	AP	-----
[62]	1984	USA	Sheep	Bacterin or CW	WOE	IM
[119]	1985	USA	Mice	Bacterin or CW	MDP, TDM, BCG and *Cpar*	IP
[35]	1986	USA	Goat	Toxoid	FIA	SC
[120]	1987	USA	Sheep	Bacterin	-----	SC
[82]	1988	Brazil	Goat	Bacterin	AP	-----
[121]	1989	Norway	Goat	Bacterin or toxoid	Levamisole	-----
[122]	1989	Norway	Goat	Bacterin + toxoid	-----	-----
[123]	1990	USA	Sheep / mice	Bacterin	MDP	IM / IP
[83]	1991	USA	Sheep / goat	Dried whole *Cp* cells	MO + AA	IM
[40]	1991	USA	Sheep	CST	BPA	-----
[60]	1991	Australia	Sheep	Toxoid or bacterin + toxoid	AH	SC
[124]	1991	Australia	Sheep	Toxoid or toxoid *Cp* + 5 Clostridial toxoids	AH / Sodium selenate	SC
[125]	1991	Australia	Sheep	Varying concentrations of toxoid *Cp* + 5 Clostridial toxoids	AH	SC
[96]	1991	Brazil	Goat	Bacterin or live attenuated *Cp*	AP (bacterin)	ID
[36]	1992	Australia	Sheep	Toxminus *Cp*	-----	SC
[98]	1994	Australia	Sheep	Toxminus *Cp* or recombinant Toxminus *Cp*	-----	OR
[38]	1994	Australia	Sheep	40-kDa antigen	AH	SC
[84]	1996	USA	Sheep / goat	Bacterin	MDP + MO	IM
[3]	1997	Australia	Mice	Live *Cp aroQ* mutant or live *Cp pld* mutant	-----	IP
[64]	1998	Australia	Sheep	Live *Cp aroQ* mutant or live *Cp pld* mutant	-----	SC
[59]	1998	Canada	Sheep	Glanvac® 6, Case-Vac® or bacterin	MDP + MO (bacterin)	SC / IM
[91]	1998	USA	Sheep	Bacterin + toxoid	AH	SC
[65]	1999	Australia	Sheep	Glanvac 6 or recombinant Glanvac® 6	MA	SC
[126]	2000	German	Goat	CW	-----	-----
[127]	2000	Australia	Sheep	Toxoid Cp + 5 Clostridial toxoids	AH / Moxidectin	SC
[97]	2002	Brazil	Goat	Liophilized live attenuated *Cp*	no	ID
[128]	2003	Perú	Mice	CST	AH	SC
[58]	2005	Egypt	Mice	Bacterin, toxoid or bacterin + toxoid	MO	SC
[99]	2006	United Kingdon	Sheep	Bacterin, rPLD, bacterin + rPLD, or Glanvac® 3	AH	SC
[61]	2007	Peru	Alpacas	Live Cp	----	SC
[92]	2007	Peru	Alpacas	CW or CST	MDP	SC
[129]	2007	Egypt	Mice	Bacterin, rPLD, or bacterin + rPLD	MO	SC
[90]	2008	Brazil	Goat	Crude CST, concentrated CST + oligodeoxynucleotide CpG, or live attenuated *Cp*	FIA	SC
[93]	2009	Brazil	Mice	Recombinant Heat-shock protein 60 (rHsp60)	FCA / FIA	SC
[130]	2010	Egypt	Sheep	Bacterin, bacterin + rPLD, Gamma-irradiated Cp + rPLD or BCG + rPLD	MO (except BCG)	SC
[131]	2010	USA	Mice	Bacterin + toxoid	-----	-----
[132]	2010	Turkey	Sheep	Bacterin + toxoid	FCA	SC
[133]	2011	Saudi Arabia	Sheep	Glanvac® 6	Olive oil	SC

Abbreviations: Immunogens - *Cp* = *Corynebacterium pseudotuberculosis*; Bacterin = *Cp* killed whole cells; CW = sonicated *Cp* cell wall; CST = filtrated culture supernatant exotoxins; Toxoid = inactivated exotoxins or phospholipase D (PLD); rPLD = recombinant PLD; Glanvac® and Case-Vac® = commercial vaccines; Toxminus = Cp with deleted *pld* gene; **Adjuvants** - WOE = Water-in-oil emulsion; MO = mineral oil; AA = Arlacel A or monooleate of manitol; AH = aluminum hydroxide; AP = Aluminum phosphate; MDP = muramyl dipeptide; TDM = trehalose dimycolate; BCG = heat-killed *Mycobacterium bovis* BCG; *Cpar* = heat killed *Corynebacterium parvum*; FCA = complete Freund's adjuvant; FIA = incomplete Freund's adjuvant; BPA = block polymer adjuvant; MA= multicomponent adjuvant; **Route of administration** - SC = subcutaneous; IM = intramuscular; IP = intraperitoneal; ID = intradermal; OR = oral.

Table 1: Summary of data from scientific works published in the last 40 years related to vaccine models against infection caused by *Corynebacterium pseudotuberculosis* in different animal models.

pseudotuberculosis. Specifically, PLD has been widely studied, as it is one of the few virulence factors known and is the best characterized protein of *C. pseudotuberculosis*. PLD has been purified, cloned and expressed in *E. coli* [32,85-87]. In some studies, the exotoxins are treated with various concentrations of formaldehyde, producing toxoids. These toxoid-based vaccines have been tested and increase antibody levels against the exotoxins as shown by ELISAs, which decreases CLA spread in sheep [35,60,88,89]. This boost in humoral immunity still only partially protected animals because vaccinated animals only had reduced numbers of lesions after challenge compared to the unvaccinated sheep. In a recent study, animals inoculated with secreted antigen associated with Freund's incomplete adjuvant and oligodeoxynucleotide containing unmethylated CpG dinucleotides (CpG ODN) showed a strong humoral response, but this inoculation could not prevent the spread of infection [90].

Some studies have used a combination of bacterins and toxoids. A product composed of killed *C. pseudotuberculosis* and PLD inactivated by formaldehyde was tested in field trials, and the serological results demonstrated the presence of antibodies against PLD as well as against cellular antigens of *C. pseudotuberculosis*. A progressive significant increase in humoral response was observed in the eighth week, but antibody titers decreased by the thirty-second week. After the challenge, the number of granulomas was significantly lower in vaccinated animals compared to controls, supporting the use of a vaccine combining exotoxin with somatic antigens. However, the immune protection was still not adequate [91].

Subunit vaccine models have also been developed in an attempt to improve the specific immunological response and promote greater rates of protection. The CP40 antigen protein from *C. pseudotuberculosis* was identified by a strategy that employs locally derived antibody-secreting cells (ASCs). ASC probes generated by culture of ASCs obtained from lymph nodes draining at the site of infection showed specificity for CP40. Sheep vaccinated twice with 100 μg per dose of CP40 in aluminum hydroxide adjuvant were protected against infection with *C. pseudotuberculosis*, with an 82% reduction in the number of infected sheep and a 98% reduction in lung lesions, suggesting that the 40-kDa antigen plays a major role in immunity to CLA [38]. Another study used an antigen of the *C. pseudotuberculosis* cell wall with muramyl dipeptide as an adjuvant and observed a reduction in CLA pyogranulomas [92]. *C. pseudotuberculosis* heat-shock protein (Hsp60) has also been tested as a vaccine candidate against CLA. The immunization of BALB/c mice with recombinant Hsp60 (rHsp60) that was expressed in *E. coli* and purified induced a significant anti-Hsp60 IgG response, with greater production of IgG1 rather than IgG2a. Cell-mediated immune responses induced by immunization were characterized by an elevated production of IFN-γ and IL-10, while IL-4 concentrations were not significantly increased. However, the subcutaneous administration of rHsp60 did not induce effective protection against intraperitoneal infection with *C. pseudotuberculosis* [93]. Other studies have identified immunodominant antigens by immunoblotting using the secreted/excreted proteins of *C. pseudotuberculosis* with immune sera from infected goats and sheep [50,94,95], but these antigens have not yet been investigated in the context of vaccines.

Experiments with live attenuated bacterial strains as vaccine models have also been performed, but the same pattern of results have been observed: evident humoral induction, varied degrees of immune protection and reduction only in the number of CLA lesions [90,96,97]. To improve the results of vaccine models based on live strains, researchers genetically engineered a mutant strain of *C. pseudotuberculosis* in which the gene for PLD was deleted by site-specific mutagenesis, named "Toxminus". When inoculated into sheep, this mutant was less virulent, causing no local granulomas (10^8 CFU / dose). With a dose of 10^{10} CFU of mutant Toxminus, small granulomas were observed in draining lymph nodes, and the mutant bacterium was isolated in only one of these granulomas. These results confirmed that PLD is essential for *C. pseudotuberculosis* survival *in vivo*. The humoral and cellular responses to this mutant were assessed and were found to be less severe than those induced by wild type strains. When animals vaccinated with Toxminus were challenged with a wild type strain (10^7 CFU), the number of granulomas and their extension were much smaller when compared with control animals. Thus, the Toxminus strain that encodes a genetically modified PLD is non-toxic and confers immunoprotection [36].

In further studies, the Toxminus group developed a new mutant, Toxminus with an additional gene encoding an inactive form of PLD. This inactive form was obtained by substituting a histidine for a tyrosine at the active site of the enzyme. Animals orally vaccinated with this mutant were 100% protected against a wild type strain. These results confirmed the importance of PLD as a protective antigen and demonstrated both the potential for developing an oral CLA vaccine and *C. pseudotuberculosis* Toxminus as a live vaccine vector [98].

Another mutant strain of *C. pseudotuberculosis*, designated aroQ, was constructed by allelic exchange [63] and tested in sheep models for its ability to act as a vaccine against a homologous challenge. The results demonstrated that aroQ mutants failed to elicit detectable specific IFN-γ-secreting lymphocytes and induced only low levels of antibodies against *C. pseudotuberculosis* culture supernatant antigens. Subcutaneous vaccination with aroQ did not protect sheep from infection with the wild-type strain, but the clinical severity of disease resulting from challenge did appear to be lower. Attempts to improve the Toxminus strain have been made by replacing a histidine residue at position 20 in the active site of PLD with a serine residue. In vaccination trials, all the immunized sheep showed evidence of low antibody titers to PLD; however, after the challenge, the titers increased significantly, indicating that animals had been sensitized by the vaccine. Nevertheless, the protection rate of the immunized animals was only 44% [65].

Recombinant DNA vaccine strategies have been pursued further. A model containing a DNA sequence for a genetically attenuated PLD and a model for the sequence that encodes the protein CTLA-4 were constructed. The goal of this study was to direct the toxin to the antigen-presenting cells (APCs) because CTLA-4 binds to B7-1 (CD80) or B7-2 (CD86) on APCs. Sheep vaccinated with the DNA vaccine for PLD protected sheep against infection, and this protection was even more pronounced in animals vaccinated with DNA for PLD associated with CTLA-4. The immune protection rates conferred by these vaccines in sheep were 56% and 70%, respectively [66]. The same DNA vaccine was tested for different routes of administration. In sheep, the vaccine was administered intramuscularly and subcutaneously. The route of administration significantly influenced the effectiveness of the vaccine, and the intramuscular route was the most efficient for this vaccine [67]. These studies opened new possibilities for improvement and innovation in veterinary immunoprophylaxis for CLA.

In a recent study, sheep were immunized with a recombinant derivative of PLD, a formalin-killed bacterin, and a bacterin supplemented with recombinant PLD. Following homologous

experimental challenge, the PLD and bacterin vaccines conferred statistically significant protection against infection and appeared to restrict the dissemination of bacteria beyond the inoculation site in the majority of animals. The most interesting observation was that the combined vaccine provided absolute protection against infection, whereby challenge bacteria were eradicated from all the vaccinated sheep. In this experiment, in addition to the experimental vaccines, a commercially available CLA vaccine that is not licensed for use in the European Union was assessed in a heterologous challenge. The vaccine conferred significant protection, although the dissemination of infection beyond the inoculation site was not restricted as it was with the previous vaccines. The authors of this study highlighted the fact that if a single commercial vaccine is chosen, it must have the capacity to protect against infection by a variety of different isolates, irrespective of their geographical origin; this must be considered when developing and proposing any vaccine model [99].

Commercial vaccines are available in various regions of the world. For example, Fort Dodge Animal Health (now Pfizer, www.pfizer.com. br) produces a vaccine called Biodectin™ that is marketed in many countries. The combined vaccines of the Glanvac™ series, which are also marketed by Pfizer, are available in Australia. Caseous D - T™ and Case - Bac™ are produced in the United States by the Colorado Serum Company (www.colorado-serum.com). A live attenuated strain of *C. pseudotuberculosis* (strain 1002) was licensed in the year 2000 for use as a vaccine in Brazil and was developed by the Empresa Baiana de Desenvolvimento Agrícola (www.ebda.ba.gov.br) in collaboration with the Health Sciences Institute of the Federal University of Bahia, Brazil. Another attenuated live vaccine, LinfoVac (Laboratórios Vencofarma do Brasil Ltda; www.vencofarma.com.br), which is licensed for use in sheep and goats, is also currently available in Brazil. Despite the fact that these vaccines have been commercially available for a long time, CLA remains prevalent.

There have been great advances in understanding the immune response against *C. pseudotuberculosis*, but improvements are still needed to develop a vaccine model for sheep and goats that promotes 100% protection. The partial protection provided by the immunization of goats and sheep with commercial vaccines may be associated with the type of immune response elicited. Protection against *C. pseudotuberculosis* is mainly dependent on an immune response that involves INF-γ production and cytotoxic T cells. A humoral response alone is insufficient to protect the animal, and a good cellular response is not achieved with inactivated vaccines.

Once CLA is a long-term chronic disease, the success of a vaccination program can to be attributed to the correct use of vaccines and good practices of animal management. These measures would allow younger animals that are already vaccinated to replace the older infected ones, thus eradicating the disease in the flock. In other words, vaccines should only be a part, though an essential part, of CLA control programs.

Serological Diagnosis of CLA

Currently, CLA diagnosis in small ruminants is based on characteristic clinical symptoms and the microbiological identification of *C. pseudotuberculosis* in material collected from abscesses. However, efficient control requires a serological diagnosis because infected animals that have no apparent symptoms are a source of infection for healthy animals [100]. To date, the isolation and identification of *C. pseudotuberculosis* by microbiological culture and biochemical testing

are still the most reliable methods of CLA diagnosis [101]. However, the puncture of the abscess for the removal of purulent material for culture infects the animal skin and the environment, representing a high risk for pathogen transmission within a herd. As such, the development of less invasive immunodiagnostic tests based on the detection of specific antibodies to *C. pseudotuberculosis* in the serum of animals has been widely promoted throughout the world [6].

Several diagnostic methods have been proposed for the diagnosis of CLA, including indirect hemagglutination [102], complement fixation [100], immunodiffusion [103], the synergistic inhibition of hemolysis [35] and PCR [104]. Numerous serological tests have been developed to detect antibodies against *C. pseudotuberculosis* in small ruminants [105]. ELISAs have been frequently used to control CLA in flocks around the world, as they detect subclinical infections in a highly specific manner [106-109]. However, no test has been found to be satisfactory alone [88]. Various antigen preparations have been assayed in ELISAs, including bacterial culture supernatants [108], cell wall antigens [7], PLD [106] and recombinant exotoxins [107]. Typically, the tests work well for goats, but they have reduced sensitivity in sheep, especially in sheep with subclinical infection or that only have internal abscesses. Most of these ELISAs are still not commercially available, and those that are have a relatively high cost [7,105,106].

As *C. pseudotuberculosis* is a facultative intracellular pathogen, cell-mediated immunity is an important component of the protective immune response [50]. Thus, a whole-blood IFN-γ assay is a promising detection tool for CLA in small ruminant flocks [75, 107, 110]. This method has been optimized, and its ability to correctly detect infected animals has been compared to ELISAs. Indeed, the IFN-γ test accurately detected *C. pseudotuberculosis* experimentally infected goats over a 363 day period with a reliability of 89.2% and in non-infected goats with a reliability of 97.1%, while a recombinant PLD-based ELISA detected *C. pseudotuberculosis* in experimentally infected goats over the same period with a reliability of 81.0% and in non-infected goats with a reliability of 97.0% [107]. A recent study developed an assay for the *in vitro* quantification of IFN-γ that is secreted in the supernatant of antigen-stimulated cells and concentrated. The samples are simple and fast to prepare, and the test is inexpensive and convenient for IFN-γ quantification. Although it is promising, the sensitivity of this method needs improvement, including efforts to improve the blood-cell culture conditions, antigen extraction procedures and stimulation protocols [75]. Sensitivity and specificity are important factors that should be considered when selecting a diagnostic assay(s) for a screening program. A test with a lower specificity can lead to false positives, whereas reduced sensitivity can lead to false-negative results. An alternative approach to the diagnosis of CLA might be the combined assessment of the *in vitro* cellular immune response to *C. pseudotuberculosis* antigens combined with ELISAs, which would fully evaluate the humoral response.

Zoonotic Potential of *C. pseudotuberculosis*

Sheep and goats are the most common animals infected within the broad spectrum of hosts in which *C. pseudotuberculosis* causes clinical disease. However, *C. pseudotuberculosis* is considered an emergent public health problem. This bacterium can produce diphtheria toxin as a consequence of lysogenization with a diphtheria phage. This has been clearly demonstrated *in vitro* [111] and has led authorities from many countries to urge their health services to be aware of diseases caused by all the diphtheria toxin-producing species of *Corynebacterium*, including *C. pseudotuberculosis* and *C. ulcerans*, in the differential

diagnosis of diphtheria. For many years, the identification of any toxigenic *C. pseudotuberculosis* required notification to communicable disease control agencies [112]. Recently, a case of toxigenic *C. pseudotuberculosis* was reported. This is the only report in the literature of the isolation of toxigenic *C. pseudotuberculosis* from a human, and this isolation occurred in the UK. The organism was isolated from the aortic root vegetation of an intravenous drug user with endocarditis; this patient had no history of animal contact, and no possible source of infection was identified [113]. In addition to the production of diphtheria toxin by lysogenic *C. pseudotuberculosis*, common strains can infect humans and cause suppurative lymphadenopathy; these events are traditionally associated with farm animal contact and contaminated dairy products. Pathologically, the infected lymph node tissue is replaced by necrotizing granulomas, while the general histological appearance resembles tuberculous lymphadenitis, cat-scratch disease and lymphogranuloma venereum, which must be considered in the differential diagnosis. A summary of the human cases of infection is presented in Table 2, including the country of origin of the report, the sex and age of the patients, the source of exposure to the pathogen (professional or occasional), the main clinical presentations of the disease and treatment.

The first human case was described in 1966; a 37-year old man from Panama that worked as a grass cutter developed an inguinal lymphadenopathy. Initially, the clinical presentation was thought to be a lymphogranuloma venereum, and the patient was treated with tetracycline for a period of 3 weeks. The lymph node was excised and presented a characteristic histological appearance of the CLA pyogranulomas that normally occur in sheep and goats. The bacterial strain was isolated and was found to produce acid from glucose, fructose, galactose, sucrose, mannose, maltose, dextrin and xylose, as well as from hydrogen sulfide, but it did not reduce nitrate to nitrite and was negative for the urease reaction [114]. Since that time, 32 new clinical cases have been reported, and the 19 human cases reported from Australia is higher than that from all other countries, with reports varying between 1 and 3 cases per country. A profile including the age, sex and source of exposure for the patients infected with *C. pseudotuberculosis* found in the reviewed literature is presented in Figure 1. Of the 33 human cases, most cases presented as axillary or epitroclear lymphadenopathy, and the main group of infected patients was men aged 21 to 40 years old that were exposed to farm animals, mostly sheep, at work. These data strongly indicate that human infection by *C. pseudotuberculosis* constitutes a bona fide zoonosis.

In most cases, lymphadenopathy has a prolonged course, and the formation of relapsing abscesses is frequent. Treatment with antibiotics is prolonged, normally lasting for more than two weeks, and typically comprises administration of intracellularly active antibiotics, such as tetracycline and macrolides. Although antibiotic therapy alone has been successful in a few cases, *C. pseudotuberculosis* is a facultative intracellular pathogen, and it is very difficult for antibiotics to reach the bacteria inside the pyogranuloma macrophages. Thus, surgical interventions including drainage or excision were often required to clear the purulent content of the thick collagen capsule. The combination of antimicrobial therapy with excision and drainage is the most successful course of treatment.

It should be noted that two specific cases of human infection due to *C. pseudotuberculosis* have involved clinical presentations other than the characteristic lymphadenopathy. In the first case, a veterinary student who worked with equines was infected by *C. pseudotuberculosis* and

presented with an eosinophilic pneumonia [115]. In the second case, a 63-year old man presented an ocular infection by *C. pseudotuberculosis*, involving a scleral buckle after retinal reattachment intervention; this case may be the first human ocular *C. pseudotuberculosis* infection [116].

The prevalence of the human disease caused by *C. pseudotuberculosis* is likely underestimated, as only 33 cases have been reported over a period of 42 years (from 1966 to 2008). In addition to this low prevalence, some cases of human exposure to *C. pseudotuberculosis* that are found in the literature were written in Russian, making them difficult to utilize. In one report, a microbiological study of 69 Russian patients with allergic annual rhinitis (AAR) and infectious rhinitis (IR) was performed and demonstrated that among the species isolated in IR, *C. pseudotuberculosis* was the predominant species associated with Streptococci [117]. In another study from Russia, the bacteriologic examination of 1589 patients showed that, aside from *C. diphtheriae*, 11% of acute upper respiratory tract infections were caused by *Corynebacterium* species. The disease processes varied significantly, presenting as bronchitis, pyelonephritis, urethritis, colpitis, dermatitis and arthritis. Although *C. pseudodiphtheriticum* and *C. xerosis* were isolated more frequently from clinical specimens, *C. diphtheriae* and *C. pseudotuberculosis* were identified as the most virulent species. *Corynebacterium* species (but not *C. diphtheriae*) were frequently isolated from clinical specimens with Staphylococci and Streptococci, and in those cases, pathogenicity and resistance to antibiotics were more pronounced; the strains isolated with other bacteria were resistant to tetracycline, penicillin, and erythromycin [118], which are the most common active antibiotics used for the treatment of intracellular bacteria. These two Russian studies provide evidence demonstrating that *C. pseudotuberculosis* is a much more widespread pathogen than suggested by the 33 published cases. Additionally, because the published human cases involved zoonotic transmission due to work with farm animals, the proper training and education of these workers is needed.

Future Directions

The two virulence determinants of *C. pseudotuberculosis*, the toxic lipid cell wall and the exotoxin PLD, have proved to be highly associated with dissemination in tissues and development of granulomatous lesions, respectively. However, knowledge of the molecular mechanisms and genetic basis of virulence of *C. pseudotuberculosis* is central to understanding the host-pathogen associations.

Because *C. pseudotuberculosis* is an intracellular pathogen, its immunopathogenesis is characterized by pyogranulomas; biologically, this represents a form of containment in the host's tissues. Data suggest that antibodies might help to protect animals against infection, but full protection by any vaccine model must provide better stimulation of cellular immunity, such as the activation of CD8$^+$ cells and the secretion of IFN-γ, to control the bacteria in an early phase of the infection process. Thus, the molecular mechanisms of infection by *C. pseudotuberculosis* need to be more thoroughly understood so that specific stimulation of cellular immune responses can be accomplished through immunization. A promising area of research is the acute phase response of the infection, as innate immunity is responsible for the early events in the control the bacterial spread; vaccines could be improved to better stimulate innate immunity.

For the diagnosis of *C. pseudotuberculosis* infection, ELISA and IFN-γ quantification are promising techniques that provide

Case nº	Year	Reference	Country of origin	Sex, age of patients	Occupation / Source of exposition	Main clinical presentation	Treatment
1	1966	[114]	Panama	M, 37	Grass cutter	Inguinal lymphadenopathy	EX and TET
2	1967	[134]	Australia	M, 28	Manager of a sheep and cattle farm	Lymphadenopathy in the inguinal, leg and thigh lymph nodes	EX, DR and PEN
3	1968	[135]	Australia	M, 24	Sheep shearer	Asymptomatic axillary lymphadenopathy	EX, PEN and TET
4	1968	[136]	Australia	M, 23	Butcher	Axillary lymphadenopathy	EX and TET
5	1974	[137]	Australia	M, 20	Sheep rancher	Axillary lymphadenopathy	EX and DR
6	1974	[137]	Australia	M, 40	Rural worker	Inguinal lymphadenopathy	EX and ERY
7	1974	[137]	Australia	F, 50	Housewife of rural worker	Asymptomatic cervical lymphadenopathy	EX
8	1979	[138]	Australia	M, 21	Abbatoir worker	Axillary lymphadenopathy	EX and CLO
9	1979	[115]	USA	M, 28	Veterinary student	Eosinophilic pneumonia	ERY
10	1980	[139]	France	M, 27	Shepherd	Axillary lymphadenopathy	EX, TET and CHL
11	1981	[140]	USA	M, 30	Raw milk ingestion	Cervical lymphadenopathy	EX, DR, PEN and ERY
12	1985	[141]	Australia	M, 18	Butcher	Epitrochlear and axillary lymphadenopathy	EX, DR, PEN, FLU, TET and ERY
13	1985	[10]	Australia	M, 41	Farm worker	Axillary lymphadenopathy	EX
14	1985	[10]	Australia	F, 29	Farm worker	Inguinal lymphadenopathy	EX, PEN and FLU
15	1986	[142]	New Zeland	M, 29	Sheep rancher	Asymptomatic inguinal lymphadenopathy	EX, PEN, CLO, FLU and ERY
16	1986	[10]	Australia	M, 29	Meat inspector	Axillary lymphadenopathy	EX and DR
17	1988	[10]	Australia	M, 22	Butcher	Axillary lymphadenopathy	EX and ERY
18	1988	[10]	Australia	M, 20	Slaughterman	Axillary lymphadenopathy	EX, DR and ERY
19	1988	[10]	Australia	F, 53	Unknown exposure	Supraclavicular lymphadenopathy	EX
20	1989	[10]	Australia	M, 40	Abbatoir worker	Epitrochlear and axillary lymphadenopathy	EX and ERY
21	1991	[143]	Belgium	NF	NF	Axillary lymphadenopathy	NF
22	1992	[10]	Australia	M, 27	Worker of a sheep saleyard	Axillary lymphadenopathy	EX, FLU and AMOX-CLAV
23	1992	[10]	Australia	M, 26	Abbatoir worker	Axillary lymphadenopathy	EX and PEN
24	1992	[10]	Australia	M, 40	Contact with sheep	Axillary lymphadenopathy	EX, ERY and FLU
25	1995	[144]	Spain	M, 34	Shepherd	Inguinal lymphadenopathy	EX and ERY
26	1997	[145]	Switzerland	M, 30	Sheep rancher and butcher (Turkey)	Epitrochlear and axillary lymphadenopathy	EX and CLA
27	1997	[146]	Australia	M, 17	Contact with farm animals	Suppurative lymphadenopathy	NF
28	1997	[147]	New Zeland	M, 22	Shepherd	Axillary lymphadenopathy	EX, DR and ATB
29	2004	[148]	Spain	F, 33	History of contact with a white rat	Cervical lymphadenopathy	AMOX-CLAC
30	2004	[149]	France	F, 63	Living in a rural area	Axillary lymphadenopathy	EX, DR, CLO, GEN, CIP and PRI
31	2005	[117]	China	M, 63	Contact only with a dog maintained as pet	Ocular infection and mucopurulent discharge	AMP, CLO, PEN and VAN
32	2006	[150]	France	F, 12	Contact with sheep during vacation in a rural area	Asymptomatic lymphadenopathy	EX, DR, IMI-CIL, RIF and OFL
33	2008	[113]	United Kingdon	NF	Injecting drug user, unknown exposure	Endocarditis	NR

Legend: EX = excision of the lymph node; DR = drainage of the lymph node content; ATB = antibiotics; TET = tetracycline; PEN = penicillin; CLO = cloxacillin; FLU = flucloxacillin; ERY = erythromycin; CHL = chloramphenicol; AMOX-CLAV = amoxicillin; CLA = clarithromycin; GEN = gentamicin; CIP = ciprofloxacin; PRI = pristinamycin; AMP = ampicillin; VAN = vancomycin; IMI-CIL = imipenem-cilastatin; RIF = rifampin; OFL = ofloxacin; NR = not reported; NF = not found.

Table 2: Summary of data from 33 previously published cases of human infection by *Corynebacterium pseudotuberculosis*.
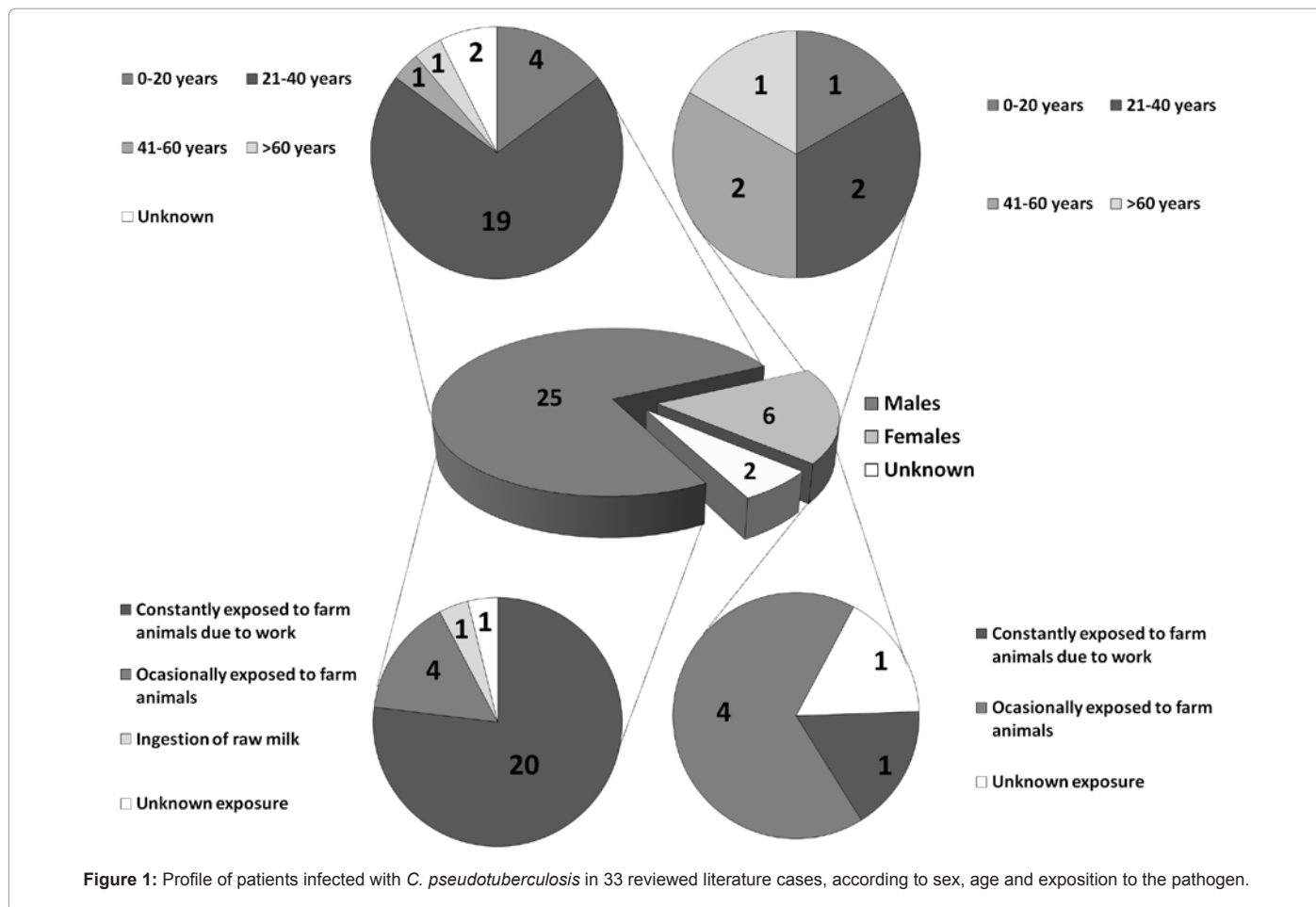
Figure 1: Profile of patients infected with *C. pseudotuberculosis* in 33 reviewed literature cases, according to sex, age and exposition to the pathogen.

high levels of reliability. With some improvement, a great variety of tests will be available to flock owners and veterinarians. However, these methodologies require a minimal laboratory structure for implementation, including microplate readers and well-trained professionals. These requirements would not allow these tests to be applied in field situations during the routine care of small ruminant flocks or in the inspection of slaughterhouses. Thus, scientists are also encouraged to develop simpler technologies that could be used in field conditions, such as a rapid immune-chromatographic test.

Because of the zoonotic potential of *C. pseudotuberculosis*, studies should be performed to characterize the presence of the bacteria in carcasses and the rates of condemnation due to *C. pseudotuberculosis*. Further, the risks to consumers who come into contact with or ingest contaminated meat or milk need to be quantified.

Acknowledgments

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