



Caseous lymphadenitis: virulence factors, pathogenesis and vaccines. Review



Maria Carla Rodríguez Domínguez ^a

Roberto Montes de Oca Jiménez ^{a*}

Jorge Antonio Varela Guerreo ^a

^a Universidad Autónoma del Estado de México. Centro de Investigación y Estudios Avanzados en Salud Animal (CIESA). Facultad de Medicina Veterinaria y Zootecnia. km 15.5 Carretera Panamericana Toluca-Atlacomulco, Toluca, Estado de México, México.

* Corresponding author: romojimenez@yahoo.com

Abstract:

Caseous lymphadenitis is a disease that affects sheep and goat production worldwide. The etiological agent is a Gram-positive, facultative intracellular bacterium called *Corynebacterium pseudotuberculosis* biovar *ovis*. The disease can occur with a cutaneous or visceral development, causing deterioration in the physical condition of the animal, as well as losses in the production of milk and meat, carcass confiscation, skin rejection and consequently, great economic losses. The study of virulence factors and pathogenesis mechanisms have made it possible to understand this disease, as well as to establish the target molecules for the development of new vaccines. There are commercial vaccines available globally; however, the protection conferred by them has not been effective in controlling the disease. Currently, the use of new technologies has allowed the obtaining and characterization of proteins with immunogenic potential for the development of new vaccines, which could be an alternative to increase protection. In the present work, the main factors of virulence of *Corynebacterium pseudotuberculosis*, their implications in the pathogenesis and the current trends in the vaccine formulations are presented.

Key words: Caseous lymphadenitis, *Corynebacterium pseudotuberculosis*, Virulence factors, Pathogenesis, Vaccines.

Received: 01/06/2020

Accepted: 08/01/2021

Introduction

Caseous lymphadenitis (CLA) is one of the diseases that most affects sheep and goat production worldwide⁽¹⁾. The etiological agent of the disease, *Corynebacterium pseudotuberculosis* biovar *ovis*, causes a chronic infection that is characterized by the formation of abscesses in cutaneous or visceral lymph nodes. This disease causes deterioration in the physical conditions of animals, decreased production of wool⁽²⁾, meat⁽³⁾ and milk⁽⁴⁾, as well as reproductive disorders⁽⁵⁾. In Australia, the analysis of three farms in the Western region, for a total of 600 animals evaluated, allowed establishing that infection by *C. pseudotuberculosis* caused a decrease in the production of greasy wool by 3.8-4.8 % and clean wool by 4.1-6.6 %. Based on the data obtained, the annual loss in wool production would be around \$17 million Australian dollars (AUD)⁽²⁾. Losses in meat production have been estimated around \$12-13 million AUD⁽⁶⁾. Other studies indicate that overall losses would be around \$30 to 40 million dollars, considering the rejection of meat and carcasses⁽⁷⁾. In Canada, it was identified that between 3 and 5 % of meat and 0.02 to 0.03 % of animals are rejected during inspection processes of producing plants⁽⁸⁾. These facts have a negative impact on exports, decreasing the possibilities of trading⁽⁹⁾.

Corynebacterium pseudotuberculosis is a zoonotic microorganism, so it also affects man, with the personnel who work in the production of small ruminants being more vulnerable⁽¹⁰⁾. CLA has been reported in several countries such as China⁽¹¹⁾, Australia⁽¹²⁾, Brazil⁽¹³⁾, Canada⁽¹⁴⁾, the United States⁽⁸⁾ and Mexico^(15,16). The spread of the disease could have been caused by sick sheep exported from Spain to South America and from Australia to North America and the Middle East⁽¹⁷⁾. The disease is poorly reported, according to the results of a survey of 264 veterinarians and 510 farmers in the UK. Only 18 % of veterinarians had seen at least one case of the disease and 45 % of farmers had noticed abscess formation in their sheep. Few producers investigate the cause of abscesses, but of 32 farms that were studied by laboratory diagnosis, 24 were confirmed with the presence of the disease⁽¹⁸⁾. The frequency of the appearance of CLA in each region or country depends mainly on the type of farm, management and control programs. Herds in extensive production, grazing and shearing

condition the appearance of the disease⁽¹⁹⁾. Antibiotic treatment, surgical interventions and the application of disinfectant solutions to external abscesses^(20,21) are not always effective options. The purulent content of abscesses can contaminate soil, food and handling equipment^(22,23) and although, *in vitro*, *C. pseudotuberculosis* exhibits sensitivity to a wide range of antibiotics⁽²⁴⁾, *in vivo*, treatment is difficult due to the intracellular nature of the bacterium⁽²⁵⁾ and the thick, dry and fibrous content of the abscess^(8,9). The World Organisation for Animal Health (OIE) considers CLA within the list of diseases that require the development of effective vaccines to reduce the indiscriminate use of antimicrobials⁽²⁶⁾. There are commercial vaccines against CLA worldwide; however, the protection conferred by these has not been efficient in controlling the disease⁽¹¹⁾. Currently, the use of new generation technologies has allowed the development of new experimental vaccines, which could be an alternative to improve protection. The new formulations seek to include molecules, the main virulence factors, that allow the activation of the humoral and cellular immune system⁽²⁷⁾. This literature review addresses the issues related to the main virulence factors, their relationship in pathogenesis and the strategies used for the development of new vaccine formulations against caseous lymphadenitis.

General characteristics: *Corynebacterium pseudotuberculosis*

The genus *Corynebacterium* includes numerous species of great importance for the medical, biotechnology and veterinary industries. The PATRIC Database had, in 2017, the report of 466 genomes and 83 species of *Corynebacteria*⁽²⁸⁾. *Corynebacterium pseudotuberculosis* belongs to the family *Corynebacteriaceae*, genus *Corynebacterium*. It has a coccobacillary morphology with an amplitude from 0.5 to 0.6 μm and 1.0 to 3.0 μm in length. This microorganism is a non-spore- or capsule-forming, non-flagellated, facultative intracellular pathogen. It has the ability to grow anaerobically, degrades galactose, maltose, L- and D-arabinose and glucose without gas production. In simple broth culture medium, growth is scarce without turbidity; however, in Brain-Heart Infusion (BHI) broth, abundant growth with yellowish-white sediment is obtained⁽¹⁾. The cell wall of the bacterium is formed by a layer of peptidoglycan composed of meso-diaminopimelic (meso-DAP) acid, arabinose and galactose as main sugars. In the reactions of the peptidoglycan II biosynthesis pathway; meso-DAP acid is the product of the reaction catalyzed by UDP-N-acetylmuramyl-tripeptide synthetase, an enzyme that has been identified in strains of *Corynebacterium*⁽²⁹⁾. Peptidoglycan in turn is covalently bound with arabinogalactans that form a lattice, which is bound to an outer layer of corynomicollic acids (22 to 36 carbon atoms), which bind to trehalose, with the end of the wall most exposed to the outside⁽³⁰⁾. This lipid structure acts as a barrier, with selective permeability mediated by integral membrane proteins called porins⁽³¹⁾. The Phospholipase D (PLD) exotoxin is considered the main virulence factor and

can be detected by a synergistic hemolysis assay against *Rhodococcus equi* or by inhibition of the β -hemolysin of *Staphylococcus aureus*. Strains of *C. pseudotuberculosis* are classified into biovar *equi* for isolates that have nitrate reductase (nitrate-positive) enzymatic activity, and biovar *ovis* for those strains that do not have such capacity (nitrate-negative)⁽¹⁾. Biovar *ovis* is the causative agent of CLA disease and has been isolated from sheep⁽¹⁵⁾, goats⁽¹⁶⁾, antelopes⁽³²⁾, cows⁽³³⁾, alpacas⁽³⁴⁾, llamas⁽³⁵⁾, ibex⁽³⁶⁾ and pigs⁽³⁷⁾.

Biovar *equi* causes the formation of abscesses in muscle tissue of the pectoral area of horses and to a lesser extent internal injuries⁽³⁸⁾, it has also been isolated from camels⁽³⁹⁾ and buffaloes⁽⁴⁰⁾. *C. pseudotuberculosis* causes infection in humans, with the existence of cases reported mainly in countries engaged in the farming of small ruminants. Clinical symptoms include axillary, inguinal or cervical adenopathies, fever and myalgia, with chronic or subacute evolution, in some cases it can also generate pneumonia⁽¹⁰⁾.

Virulence factors

Virulence factors are the structures and molecules that give the bacterium the ability to be pathogenic. The acquisition of genes by horizontal transfer has been transcendental in the evolution of the pathogenicity of bacteria; since the functions of the acquired genes have allowed it to adapt to different environmental conditions, including survival in different niches during infection to the hosts. Most of the virulence genes of *C. pseudotuberculosis* are clustered in the genome in regions called pathogenicity islands (PAIs). In *C. pseudotuberculosis*, 16 PAIs have been identified, called PiCp, where the presence of a transposase gene in PiCp1 possibly allowed the incorporation of PAIs into the genome^(29,41). These regions contain several genes involved in adhesion, invasion, colonization, spread within the host, survival inside infected cells and evasion of the immune system. PiCp sequences have a high level of intra-biovar similarity (82–100 %) in *ovis* strains, which exhibit from 78 to 91 % similarity with respect to biovar *equi*. However, biovar *equi* strains contain large deletions and a lower level of intra-biovar similarity (77–88 %) and also compared to PiCp of biovar *ovis* (62–74 %)⁽⁴¹⁾.

Corynomicolic acids

Corynomicolic acids are part of the outer layer of the cell wall of the bacterium, which constitutes a protective and permeable barrier. The binding of these to trehalose molecules leads to the formation of curved structures, which blocks the access of molecules (antibiotics

or lysozymes) to the peptidoglycan, conferring mechanical protection^(30,31). Unlike the linear fatty acids of phospholipids, corynomicolic acids are β -hydroxy-branched fatty acids, which require carboxylation and condensation of two fatty acids for synthesis. The AccD2 and AccD3 enzymes are carboxylases widely conserved in the family *Corynebacteriaceae*, involved in the generation of intermediates for the synthesis of corynomicolic acids. Other enzymes involved in synthesis are AccD1, for the elongation of the mycolic acid chain, and FadD, an AMP ligase⁽²⁹⁾. Inoculation of corynomicolic acids in sheep showed that haptoglobin (Hp) increased its concentration three times, as well as twice the levels of serum amyloid A (SAA), proteins that indicate inflammation and acute infections⁽⁴²⁾. These results indicate their virulent potential, since on their own, they are able to induce reactions of inflammation in the host, which contributes to the formation of granulomas.

Phospholipase D, PLD

The Phospholipase D exotoxin is considered the main virulence factor of *C. pseudotuberculosis*. The *pld* gene was identified and sequenced in 1990, is part of the pathogenicity island PiCp1 and encodes a 31.4KDa protein⁽⁴³⁾. Comparison of the sequence of the PLD protein of *C. pseudotuberculosis* revealed that it has greater similarity with Phospholipase A2; however, PLD does not belong to the family of phospholipases because it lacks the HKD motif conserved in this family⁽⁴³⁾. PLD is classified as a Sphingomyelinase D (SMasA; EC 3.1.4.41), also known as sphingomyelin phosphodiesterases D or phospholipase D (PLD), which catalyzes the hydrolytic cleavage of sphingomyelin to produce choline and ceramide 1-phosphate or choline and lysophosphatidic acid (LPA)⁽⁴⁴⁾. Compounds derived from sphingomyelin degradation cause platelet aggregation, endothelial hyperpermeability, and pro-inflammatory responses⁽⁴⁵⁾. The enzymatic action of PLD hydrolyzes sphingomyelin, the main component of cell membranes, which leads to an alteration of the morphology of the target membrane. PLD contributes to the spread and persistence of the bacterium inside macrophages⁽⁴⁶⁾. The *pld* gene presents a widely conserved sequence in the strains of *C. pseudotuberculosis*, and when this is modified, the ability to produce the disease is hindered⁽⁴¹⁾.

Endoglycosidase, CP40

The CP40 protein is encoded by a gene with an open reading frame of 1,137 bp, which is located downstream of the *pld* gene in PiCp1. It was described as an enzyme with serine protease activity⁽⁴⁷⁾, but in another study, the analysis of the sequence allowed its grouping

closer next to endoglycosidases and further away from serine protease sequences⁽⁴⁸⁾. In this work, it was proposed that its enzymatic activity is endoglycosidase, mediator of the hydrolysis of glycosidic bonds, proteins of the GH18 family, similar to the EndoE domain belonging to *Enterococcus faecalis*. GH18 enzymes contain a conserved consensus sequence motif (LIVMFY) - (DN) -G- (LIVMF) - (DN) - (LIVMF) - (DN) -X-E, where terminal glutamic acid is essential for enzymatic activity. By aligning the GH18 active site in CP40, its similarity to the EndoE and EndoS enzymes was established, only with changes in one or two amino acids respectively. The function as a virulence factor has been associated with the demonstrated ability *in vitro* to degrade the Fc region of IgG antibodies. CP40 endoglycosidase does not hydrolyze glycans in bovine and caprine IgG, while sheep IgG is partially hydrolyzed and equine IgG completely. The analysis was also performed with subclasses of human IgG, presenting activity in all and partially in IgG4. There was no detectable enzymatic activity in other glycoproteins, including some of the other immunoglobulin isotypes (IgA, IgD, and IgE)⁽⁴⁸⁾.

Secreted proteins PLD and CP40

According to various reports, proteins exported or secreted by bacteria are actively involved in the infection process⁽⁴⁹⁾. For this reason, the expression and secretion of the PLD and CP40 proteins have been highly studied. The development of an experimental infection showed by immunoblot that the production of antibodies was directed 88 % to the recognition of proteins of 30-31Kda (PLD) and in 75 to 88 % towards proteins of 38-41KDa (CP40), range in which are both proteins⁽⁵⁰⁾. The attenuated strain 1002, after several passes in a mouse model, was able to reverse the virulence. The analysis by mass spectrometry allowed the identification of the PLD and CP40 proteins only in the reactivated strain 1002, which indicates the participation of these proteins in the virulence⁽⁵¹⁾. On the other hand, through real-time PCR, the expression of several genes involved in virulence was identified *in vitro* and *in vivo*, including *pld* and *cp40*. This analysis made it possible to verify that, in the strains isolated from lymph nodes, the expression of these genes was higher compared to the strains obtained from *in vitro* cultures⁽⁵²⁾.

Virulence factors involved in iron acquisition

In PiCp1 is the operon (*fag ABCD*)⁽²⁹⁾, composed of four genes, *fagA*, *fagB*, *fagC*, *fagD*, that are located downstream of the *pld* gene. These genes respectively encode an integral membrane protein, an iron-transporting enterobactin, an ATP-binding cytoplasmic

membrane protein, and an iron-binding siderophore protein. FagA was identified as a membrane-associated protein with pathogenic potentialities, by mass spectrometry analysis of proteins expressed in a strain of *C. pseudotuberculosis* grown with animal serum⁽⁵³⁾. The culture of *C. pseudotuberculosis* in media with iron chelators (dipyridyl) caused the decrease in a logarithm order in the count of colony-forming units (CFUs), compared to bacteria grown in iron-enriched media. The evaluation of the transcriptional response of *C. pseudotuberculosis*, with iron restriction, allowed identifying the negative regulation of genes involved in the energy metabolism of the Krebs cycle (*sdhC*, *sdhB*, *lpd*), ATP production (*atpF*, *atpH*), pyruvate metabolism (*lpd*), oxidative phosphorylation (*qcrC*, *qcrA*, *qcrB*, *ctaC*, *ctaF*, *ctaE*, *ctaD*), ribosome processes, transport (*rplJ*, *rplL*, *rplM*, *rpmA*, *rpsC*, *rpsI*, *rpsL*, *rpsM*) and EF-G and EF-Ts elongation factors associated with the translation of mRNA (*fusA*, *tsf*). The gene analogous to *dtxR* of *C. diptheriae* was identified, with 79 % similarity in sequence, which encodes an iron-binding dependent protein, which acts as a regulatory factor for more than 40 genes⁽⁵⁴⁾. In PiCp3 and PiCp4, the genes belonging to the operon *ciuABCDE* involved in iron absorption, transport and biosynthesis of siderophores have been studied⁽²⁹⁾.

TetA Protein

In PiCp2, the *tetA* gene encodes a tetracycline efflux transporter protein that protects against the action of this antibiotic and confers resistance to the bacterium. The *tetA* gene is often carried by transmissible elements such as plasmids, transposons and integrons and has been identified in *C. pseudotuberculosis*⁽²⁹⁾.

Virulence factors for macrophage infection

On the pathogenicity island PiCp2 are the *potG*, *sigK* and *dipZ* genes, which respond to the mechanisms responsible for the intramacrophagic lifestyle of *C. pseudotuberculosis*. The *potG* gene encodes an ATP-binding membrane protein that provides energy for absorption of putrescine (polyamine) from the periplasmic space, it functions as a putrescine transporter system⁽²⁹⁾. Putrescines are polyamides produced by macrophages that induce decreased production of reactive nitrogen species and synthesis of pro-inflammatory cytokines. The *dipZ* gene has been identified in the phylum Actinobacteria, is regulated by sigK⁽⁵⁵⁾ and is activated during macrophage infection by *Mycobacterium tuberculosis*. In PiCp4, the presence of the gene that encodes the Sigma factor confers the ability to protect against oxidative stress, specifically against the action of nitrogen intermediate products, produced

by macrophages⁽⁵⁶⁾. The FCR41 strain of *C. pseudotuberculosis* was used in the study of genes related to pili synthesis. The structure of the pili is composed of the major pili SpaA and SpaD; the minor pili SpaB and SpaE; and the pili type, SpaC, SpaF. A complete pili structure or even the minor pili can make initial contact with host cell receptors to facilitate the entry of microorganisms. In this strain, the *spaC* gene was identified, which encodes a protein responsible for anchoring the pili to the cell wall, which can allow initial contact with the cell receptors, to then facilitate intracellular invasion. It also presented the *namH* gene, which encodes an extracellular neuraminidase, which catalyzes the elimination of sialic acid groups present in a wide variety of glycoconjugates of the extracellular matrix of the host cell, which favors adherence to cells. In FCR41, the *sodC* gene was detected, which encodes a superoxide dismutase, an enzyme anchored to the membrane with extracellular domain, which eliminates oxygen free radicals, products of respiratory burst in macrophages⁽⁵⁷⁾.

Resistance and adaptation virulence factors

Chaperone proteins (HSPs) are highly conserved and are expressed under heat stress, as well as nutrient reduction, hypotaxia, breakdown of metabolism and other cellular processes. In *C. pseudotuberculosis*, Hsp10 (*groES*) and Hsp60 (*groEL*)⁽⁵⁸⁾ have been studied. In strain 1002, resistance to various types of abiotic stress, such as acidity, high temperatures and osmotic stress, associated with the presence of these proteins, was evaluated⁽⁵⁹⁾. The *hspR* gene encodes the regulatory factor of the expression (repressor protein) of the operon with the genes *dnaK*, *grpE*, *dnaJ* and *hspR*, which encode heat shock proteins. In the absence of stress, the HspR protein attaches to an inverted repeat sequence that represses the promoters responsible for controlling the expression of the Hsp operon. In another study, the separation of proteins by two-dimensional electrophoresis allowed the identification of 11 new extracellular proteins, 3 with unknown functions and 8 related to the elongation factor Tu, GroEL (HSP60), enolase, glyceraldehyde-3-phosphate dehydrogenase and superoxide dismutase (SodA), which depend on a non-classical secretion method via SecA. Both SecA genes (SecA1 and SecA2) were identified in the strains studied, possibly involved in the secretion system of *C. pseudotuberculosis*⁽⁶⁰⁾. The study of the virulence factors of *C. pseudotuberculosis* as candidate molecules for the development of more powerful and effective vaccines is still being continued.

Pathogenesis and immune system evasion mechanisms

The cutaneous manifestation of CLA is characterized by the formation of abscesses in subcutaneous lymph nodes, which are visible and palpable through the skin and their location depends on the point of entry of the microorganism. Lesions can appear as organized abscesses, with inflammation, fibrous encapsulation, overlapping hair loss and eventual rupture, resulting in the discharge of purulent content. In the visceral form, abscesses occur in the internal lymph nodes, as well as in the lungs, liver and kidneys, causing deterioration in the organic condition of the animal towards the development of a chronic course⁽⁶¹⁾. In atypical forms of the disease, macroscopic lesions do not correspond to caseous nodes, with neonatal toxemia or icterohemoglobinuria of newborns, arthrosynovitis, endometritis, mastitis and orchitis being described⁽⁶²⁾.

The infection begins with the entry of the bacterium through skin lesions generated during the handling of sheep, such as tail cuttings, ear marking, castration, shearing or in some cases, lesions generated during feeding with spiny fodder that damage the oral mucosa. Sanitary baths also contribute to infection, favoring the entry of the microorganism through small wounds of the skin⁽¹⁾. Primary infection occurs at the site of entry of the bacterium, with hematogenous and lymphatic spread forming abscesses in the lymph nodes closest to the site of infection (parotid, submandibular, prefemoral, prescapular, popliteal or mammary). Then a secondary infection occurs with the formation of abscesses in lymph nodes (thoracic, bronchial and mediastinal) and various organs^(63,64). In sheep, the morphological appearance of the abscessed nodes is the characteristic of an onion layer as they present a distribution in fibrous concentric layers separated by caseous material. In goats, the affected nodes usually form a dry uniform purulent paste. This difference could be due to the nature of phagocytic enzymes, being of greater lytic activity in goats than in sheep^(8,9).

The mechanisms involved in the adherence and intracellular survival of *C. pseudotuberculosis* in non-phagocytic cells are still being studied by various researchers. In *in vitro* studies, *C. pseudotuberculosis* was able to adhere to and invade the FLK-BLV-044 fibroblast line of sheep kidney embryonic cells, with cell replication for 24 h and bacterial viability of 120 h, with a positive correlation between the rate of adhesion and invasion⁽⁶⁵⁾. These results suggest that the establishment of infection, as well as persistence, may be favored by intracellular infection in tissue of the site of entry of the microorganism and not only by infection of phagocytic cells. *C. pseudotuberculosis* is phagocytosed by macrophages that are recruited to the site of infection, and it has been shown that they have the ability to remain viable within these for up to 72 h, evading the mechanisms of elimination of pathogens presented by macrophages⁽⁶⁶⁾. Macrophages infected with *C. pseudotuberculosis* activate the production of reactive oxygen intermediate (ROI) compounds, which cause

damage at the DNA level. The binding of bacteria to the receptors of the macrophage phagosome membrane causes the so-called respiratory burst that favors the production of NADH. Before the lysosome fuses with the phagosome, in the latter, a reduction of molecular oxygen (O_2) catalyzed by NADPH-oxidase present in the phagosome membrane occurs. The resulting superoxide anion (O_2^-) is toxic to the bacterium, but in turn gives rise to other short-lived toxic radicals, such as hydrogen peroxide (H_2O_2), hydroxyl radical (OH), and singlet oxygen (O^1_2). When the lysosome fuses with the phagosome, the myeloperoxidase enzyme is released, which acts on peroxides in the presence of halides (I^- and Cl^-), to produce highly toxic and long-lived halogenated compounds (hypohalides): hypochlorous acid (ClOH) and hypoiodous acid⁽⁶⁶⁾. However, *C. pseudotuberculosis* has developed resistance mechanisms to protect against O_2 -free radicals.

Superoxide dismutases (SODs) constitute a family of three metalloenzymes (FeSOD, MnSOD and CuZnSOD) with different intracellular localization and distribution, which catalyze the conversion of superoxide radicals into H_2O and O_2 . The presence of Mn/FeSODs in *C. pseudotuberculosis* has been verified and the phylogenetic analysis allowed establishing the evolutionary differences and similarities of the sequences of this enzyme in hosts of the sheep, goat, bovine, equine and human species, as well as with different species of *Corynebacterium*. This would explain the ability of the same strain of *C. pseudotuberculosis* biovar *ovis* to remain in macrophages of different types of hosts⁽⁶⁷⁾. *C. pseudotuberculosis* presents the catalase enzyme⁽¹⁾ that provides protection against the action of H_2O_2 , since they decompose it into H_2O and O_2 . Also, the intermediate products of the enzyme during the dismutation reaction can bind to NADPH oxidase, which functions as a regulator of enzymatic activity and decreases the formation of other oxidative stress products such as hydroxyl radical and singlet oxygen (O^1_2).

Also, in macrophages, reactive nitrogen products (RNI) act as another mechanism of elimination of pathogens. The *in vitro* study of sigma factor mutant strains of *C. pseudotuberculosis*, they were more susceptible to concentrations of nitric oxide, so it is proposed that its presence protects against this type of oxidative stress⁽⁵⁶⁾. The presence of corynomycolic acids gives *C. pseudotuberculosis* mechanical and possibly biochemical protection, allowing it to resist digestion by hydrolytic enzymes present in lysosomes and the action of antimicrobial proteins⁽⁶⁶⁾. Inoculation of corynomycolic acid extracts, in female goats, caused hemorrhage, congestion, degeneration, necrosis, edema and leukocytic infiltrations in reproductive organs and associated lymph nodes, as well as increased the concentration of estrogenic hormones⁽⁶²⁾. They have also been associated with decreased testosterone and loss of semen quality, with increased production of pro-inflammatory cytokines⁽⁵⁾. PLD exotoxin catalyzes the dissociation of sphingomyelin, an important component of cell membranes, whose hydrolysis causes cell lysis, increasing vascular permeability, with the consequent formation of edema⁽¹⁾. PLD acts directly on endothelial

cells around the point of infection and on macrophages once the bacterium has been phagocytosed. The action of this toxin facilitates colonization, regional and systemic spread of the bacterium, with the generation of abscesses in the lymph nodes⁽⁶⁶⁾.

Bacteria not controlled by the abscess wall enter the capillaries and form colonies that occlude the blood vessels, generating ischemia that, together with toxins, destroy the cells of healthy tissue, increasing the necrotic mass. Viable bacteria spread through the lymphatic vessels and penetrate other lymph nodes and blood vessels, reaching different organs where abscess formation is repeated. This behavior originates the clinical manifestations of the visceral type of the disease, which affects internal lymph nodes and organs, especially lungs and liver^(61,64).

C. pseudotuberculosis is released from inside the cells as a result of a process that leads to the death of phagocytes. Although the specific mechanisms are not yet clear, it is proposed that cell death of macrophages is not induced by autophagy or apoptosis. Studies carried out *in vitro* by infection of the macrophage line J774 with *C. pseudotuberculosis*, allowed determining that the levels of protein I associated with light chain 3 microtubules (autophagy mechanism) and the activity of caspase-3 (apoptosis mechanism) remained stable without variation in infected cells⁽²⁵⁾. In other studies, necrosis has been favored instead of apoptosis, in macrophages infected with *C. pseudotuberculosis*, causing degenerative changes such as the rupture of the plasma membrane, alterations in the mitochondria, changes in the nuclear envelope, dilation of the nuclear envelope and membrane of the rough endoplasmic reticulum and formation of vesicles in the cytoplasm^(68,69).

Another characteristic of the disease is the formation of pyogranulomas, as a result of uncontrolled bacterial growth within macrophages, the host tries to restrict and limit infection through the formation of these structures. Immunohistochemical studies on the cellular composition of lung lesions in sheep infected with *C. pseudotuberculosis* have revealed a predominance of large macrophages in the walls of the abscess and surrounding the pulmonary parenchyma, with expression of molecules of the major histocompatibility complex (MHC) class II. T lymphocytes were prominent in the lesions, while B lymphocytes and granulocytes comprised a smaller portion in the cell infiltrates. Within the encapsulated lesions, lymphocytes and MHC class II cells were found in the center of the necrotic mass. Surrounding this region, CD5⁺ cells, as well as CD4⁺ and CD8⁺ cells distributed through the lymphatic tissue, were identified. Generally, in immature caseous lesions, CD4⁺ lymphocytes are found and in more developed lesions, the concentration of CD8⁺ cells is predominant, which is related to the mechanism of the immune system to combat the spread of infected macrophages^(70,71).

In goats, the histopathological changes observed in the reproductive tract and lymph nodes after experimental inoculation with *C. pseudotuberculosis* revealed leukocytic infiltration, as well as generalized congestion, degeneration, infiltration of stromal cells and necrosis in the ovaries⁽⁷¹⁾. The study of the response of the immune system in an experimental model allowed establishing that the humoral response begins between day 6 and 11 post-infection. From day 5 of infection, the expression of IFN- γ with values from 0.5 to 1.0 (DO450) occurs, followed by a second production from day 16 post-infection with maximums from 2.5 to 3.0, high values that keep until day 42-56 of the infection, where the response begins to decline. Primary production of IFN- γ has been associated with the innate response that involves NK cells, while the secondary response of longest duration is associated with the acquired immune response with the involvement of T cells⁽⁷⁰⁾. The production of pro-inflammatory cytokines TNF- α and IL6 occurs at the inoculation site, while IFN- γ is found in drained lymph nodes⁽⁷²⁾.

Commercial vaccines

Most commercial vaccines available for CLA are composed of polyvalent formulations, presenting a combination of antigens of several pathogenic agent including the PLD exotoxin, considered the antigen with the highest immunogenic capacity for *C. pseudotuberculosis*^(11,27). They have been employed for several decades, however, they are not yet available in all small ruminant producing countries, including Mexico.

The Glanvac 3 vaccine (Zoetis, London)⁽⁷³⁾ combines toxins of *Clostridium perfringens* Type D, *Clostridium tetani* and *C. pseudotuberculosis* and evaluated in sheep from the United Kingdom, it reported that only 20.8 % of the total of six animals vaccinated and challenged with a virulent strain presented lesions from which the bacterium was isolated. The Glanvac 6 vaccine (Zoetis, West Ryde, Australia) has a multicomponent formulation that includes toxins of *C. pseudotuberculosis*, *Clostridium perfringens* type D, *Clostridium tetani*, *Clostridium novy* type B, *Clostridium septicum* and *Clostridium chauvoei*. This vaccine reduces the clinical manifestations of the disease and the development of lung lesions^(8,13). In Australia, it is administered to both sheep and goats, and several field assays have shown variable protection rates, with values ranging from 25 to 90 % of the total herd. In 1995, the average prevalence of CLA in adult sheep in vaccinated herds was 97 % in New South Wales, 91 % in Victoria and 88 % in Western Australia. By 2003, the estimated average prevalence of CLA in the adult sheep population had declined to 29 % in New South Wales, 26 % in Victoria and 20 % in Western Australia; and from 26 % overall to 5.9 % in 2009. In addition, the study allowed establishing that only 43 % of producers used the vaccine and of these, only 12 % adequately followed the manufacturer's instructions^(6,12). However, these vaccines

of the Glanvac series do not prevent infection and present adverse reactions with the formation of cutaneous granulomas at the injection site, both in sheep and goats, with anaphylactic shock in the latter^(7,8). In Canada in 1998, an evaluation of the efficacy of Glanvac 6 was carried out compared to the commercial Case-Bac vaccine (Colorado Serum, USA), and an experimental vaccine composed of muramyl dipeptide. The Glanvac 6 vaccine and the experimental vaccine had a higher antibody titer than Case-Bac for 6 to 12 months; however, Glanvac 6 caused a high number of allergy manifestations at the inoculation site⁽⁷⁴⁾.

The Caseous D-T vaccine (Colorado Serum, USA) composed of toxins of *Clostridium perfringens* type D, *Clostridium tetani* and inactivated whole cultures of *C. pseudotuberculosis* in combination with PLD has been used in the United States, demonstrating that it helps to decrease the presence of internal and external abscesses, although with side effects such as mild lameness (pain) in lambs and lethargy in a high percentage of mature animals. On the other hand, the Case-Bac vaccine (Colorado Serum, USA), composed of PLD toxin, has been used mainly in sheep. This vaccine also causes adverse reactions at the inoculation site, lethargy, stiffness and fever, these symptoms being more severe in goats, including manifestations of ventral edema, ataxia and seizures, which leads to this prophylactic not being approved for use in this species. The use of this vaccine in sheep reduced the formation of abscesses, where only 10 of a total of 18 animals presented external abscesses compared to the control group where all developed these lesions. Moreover, only 2 of the 18 vaccinated sheep had internal abscesses, while 9 out of 10 control sheep had internal abscesses^(8,9).

In Spain, the company Zoetis sells the Biodectin™ vaccine, which is composed of six antigenic fractions: *Clostridium septicum*, *Clostridium novyi* Type B, *Clostridium tetani*, *Clostridium perfringens* Type D, *C. pseudotuberculosis* and *Clostridium chauvoei*, aluminum hydroxide, Thiomersal and Moxidectin (compound with antiparasitic activity)⁽⁷⁵⁾. There are also commercial vaccines made from live attenuated strains, such as LinfoVac (Laboratorios Vencofarma do Brasil), developed by the Company Baiana de Desenvolvimento Agrícola (www.ebda.ba.gov.br) in collaboration with the Institute of Health Sciences of the Federal University of Bahia, which contains the live attenuated strain 1002 of *C. pseudotuberculosis* and its use is authorized in Brazil. Experimental studies in a mouse model indicated that the protection conferred by this vaccine is 80 %⁽¹⁰⁾.

The protection provided by commercial vaccines is associated with the production of anti-exotoxin PLD antibodies, which protect against tissue damage and the spread of the microorganism. However, they confer partial protection, since these vaccines do not favor the activation of the cellular immune response, mainly cytotoxic T-type necessary to eliminate intracellular bacteria. For this reason, different groups of researchers have worked on the development of experimental vaccines to improve protection.

Experimental vaccines

Inactivated vaccines and toxoids

Inactivated vaccines are composed of non-viable whole cultures of the bacterium or inactivated toxins, either by chemical or physical methods. In these formulations, the microorganism is dead, so they do not confer danger of development of the disease; however, the response is mainly humoral, less intense, requires high concentrations of the microorganism and several doses. They are not subjected to any purification procedure, so they contain all the biochemical components of the bacterium, which are more reactogenous and can produce adverse effects. The protein precipitate of a strain of *C. pseudotuberculosis* isolated from alpaca in Peru was evaluated in a group of 20 BALB/c mice, where it induced protection from challenge with 10^4 CFUs of a virulent strain of *C. pseudotuberculosis*. The vaccine reduced the toxic effects caused by the bacterium, which was observed with the decrease in the number and size of abscesses in the animals of the vaccinated group (40 % affected) compared to the multiple abscesses of greater size at the subcutaneous level and in kidney and liver in the animals of the control group (95 % affected)⁽⁷⁶⁾.

Vaccine formulations based on 250 - 500 mg/ml of cell wall and 133-265 mg/ml of PLD toxin, all supplemented with 20 mg/ml of muramyl dipeptide as an adjuvant, were evaluated in alpacas, subjected to the challenge with 10^6 CFUs of a virulent strain of *C. pseudotuberculosis*. The animals vaccinated with the highest dose of PLD did not show abscesses, unlike the vaccinated group with the lowest concentration, where the formation of abscesses at the inoculation site and in renal lymph nodes was observed. Formulations that included cell wall showed a lower degree of protection, with the formation of superficial and internal abscesses. The results suggest that the concentration of PLD toxin may influence the protective capacity, being dose dependent on the immunization of alpacas⁽⁷⁷⁾.

The degree of protection conferred by vaccination with PLD toxin adsorbed in aluminum hydroxide gel (Group 1) or its combination with toxins of *Clostridium perfringens* D, *Clostridium novyi* B, *Clostridium tetani*, *Clostridium septicum* and *Clostridium chauvoei* (Group 2), as well as toxins of *Clostridium* spp., PLD and 1.2 mg/ml of sodium selenate (Group 3); it was evaluated in an ovine model, challenged with 1.3×10^8 CFU of a virulent strain of *C. pseudotuberculosis*. The percentage of affected animals in group 1 was 10.5 % (4 of 38 animals showed a superficial lesion), for group 2 7.9 % (3 of 38 animals showed a superficial lesion) and 8.3 % in group 3 (2 of 24 animals, each with a superficial lesion and at the lung level), in contrast to the results obtained in the control group of unvaccinated animals with an impact of 51.5 % (17/33), with 60 lesions at the lung level and 16 in

carcasses, with 4.5 lesions per animal. The results indicated that there was no reduction of protective potency as a result of the combination of PLD with *Clostridium* antigens⁽⁷⁸⁾.

The efficacy of four non-commercial vaccines based on PLD as an antigen was evaluated in sheep challenged with a virulent strain. The levels of superoxide ions were determined as a non-specific immune response, these being elevated in the group vaccinated with PLD + inactivated bacterium, followed by the group vaccinated with PLD Toxoid. Lysozyme activity was higher in the group vaccinated with PLD + inactivated bacterium, followed by PLD Toxoid, PLD + commercial Covexin8 vaccine and a local experimental vaccine. The group vaccinated only with PLD showed a marked positive response of lymphocyte proliferation compared to the rest of the groups. The results indicated that PLD stimulated the specific and non-specific cellular immune response⁽⁷⁹⁾.

Four different antigenic extracts obtained from the attenuated strain T1 were evaluated in goats of Canindé breed, for the study of the humoral and cellular immune response. Animals in group 1 (immunized with 0.5ml of the culture supernatant of the T1 strain in 1:1 ratio with Freund's incomplete adjuvant, FIA) and group 3 (immunized with 100 mg of extracellular concentrate, 250 mg of CpG oligodeoxynucleotides and 0.5 ml of adjuvant FIA) showed the highest levels of antibodies and IFN- γ after immunization, as well as post-challenge with 10^5 CFUs of the virulent strain VD57, compared to groups 2 (1ml of suspension of 2×10^6 CFU/ml strain T1), group 4 (formulation of group 3 without FIA) and control group. Only 25 % of the animals in group 1, 33.3 % in group 2 and 22.2 % in group 3 did not develop lesions, in group 4 and 5, 100 % of the animals developed some type of lesion⁽⁸⁰⁾. These results show that the production of specific anti-PLD antibodies only decreases the spread of the bacterium and the appearance of lesions in tissues other than the inoculation site, not controlling the infection. Bacterin or toxoid vaccines contribute to reducing the clinical manifestations of the disease, being more efficient in sheep than goats, although in neither species is it possible to control the infection, leaving a percentage of affected animals that can spread the disease.

Attenuated vaccines

Attenuated vaccines have live immunizing agents that can replicate in the body without causing disease, since they lack certain structures or molecules that decrease their virulence. In principle, they confer a very intense and long-lasting immune response, since they give rise to an infection similar to the natural one, but they constitute a risk, since in some cases the virulence can be reversed. The first experimental attenuated vaccines for CLA used a strain called Toxminus, whose *pld* gene was modified by site-specific mutation. The

necropsy of the animals vaccinated with 10^5 to 10^7 CFUs of the attenuated strain Toxminus allowed observing that no abscesses formed in the animals challenged with 10^6 CFUs of a virulent strain, compared to the control group where abscesses of 2.5 cm developed in popliteal ganglia. However, the vaccine produced an undesirable abscess at the inoculation site, the antibody titer in the groups vaccinated with 10^5 to 10^7 CFUs was similar, so the response was non-dose dependent and the virulent challenge strain induced a superior antibody response at weeks 5 and 9. There was also a reduction in the ability of the Toxminus strain to remain in the host, due to the absence of PLD, an antigen that favors persistence, in addition to highly activating the humoral immune response⁽⁸¹⁾.

In another study, the Toxminus strain was transformed with a plasmid containing the *pld* gene modified to obtain the exotoxin with a change of histidine for a tryptophan at position 20, which eliminates enzymatic activity. Immunization with the orally administered Toxminus strain induced a predominant humoral response of IgG1 type, while IgG2 isotype levels were higher in subcutaneously vaccinated sheep. Th1 cells are responsible for cell-mediated cellular immunity, they produce IFN- γ , IL-2, and tumor necrosis factor beta (TNF- β), cytokines that activate macrophages and complement the activation of B lymphocytes to produce antibodies of the IgG2 isotype. On the other hand, Th2 clones secrete IL-4 and preferably induce the production of IgG1, IgA and IgE in B cells. Consequently, with the results obtained, the orally vaccinated animals did not present significant levels of protection, because the increase in IgG1 is indicative of the absence of a Th1 response, which is essential for the activation of cytotoxic T cells, essential for the elimination of intracellular pathogens, such as *C. pseudotuberculosis*. The incidence and degree of abscess formation were very low (abscesses of 0.2 cm and 1 cm), occurring only in two animals at the site of inoculation of the virulent strain for the challenge. In the group of animals vaccinated with the non-modified Toxminus strain, 50 % of the sheep developed abscesses, as well as 66 % of animals in the unvaccinated control group. The Toxminus strain did not allow for elevated PLD expression and evidence of the excretion of the live attenuated bacterium through feces was found⁽⁸²⁾.

The CZ171053 strain mutated in the *ciuA* gene, by means of the transposon-TnFuZ system, presented a reduced ability to survive *in vitro* within the macrophages of the J774 cell line. The immunization of BALB/c mice with this attenuated strain allowed the survival of 80 % of the animals challenged with 10^6 CFUs of the virulent strain MIC-6. These results suggest that the CZ171053 strain could be evaluated as a live attenuated vaccine in the target hosts of the disease.

The behavior of the humoral and cellular immune response was evaluated in BALB/c mice inoculated with 10^7 CFUs of the attenuated strain T1. An increase in the titer of IgG1 and IgG2 was observed, no lesions characteristic of the disease were shown and the culture of spleen cells, stimulated *in vitro* with antigens secreted by T1, presented a greater proliferation compared to cells stimulated with intracellular antigens⁽⁸³⁾.

DNA vaccines

Advances in molecular biology techniques have allowed the development of new generation vaccines, among which are naked DNA vaccines. These vaccines are only DNA (plasmids with the genes of interest), they do not have envelopes or protein structures, so the route of administration, the dose, and re-immunization are very important, since they are factors that influence the potency and type of immune response. The disadvantage of this type of vaccine lies in the ability to express the antigen of interest, since in most cases, the particles have a low adsorption and the quantity of plasmids that are introduced into the cells is limited.

The design of a plasmid carrying the gene encoding the extracellular domain of bovine CTLA-4 fused to the inactivated *pld* gene (boCTLA-4-HI γ - Δ PLD) was evaluated as a naked DNA vaccine in sheep. CTLA-4 binds with high affinity to B7 membrane antigen in antigen-presenting cells (APCs), improving the humoral immune response. Although the antibody titer increased significantly, the protection of immunized sheep was partial against the experimental challenge with a virulent strain⁽⁸⁴⁾. Different immunization routes were evaluated: intramuscular, subcutaneous and gene gun bombardment. The maximum levels of total IgG antibodies were 12×10^3 in the group vaccinated intramuscularly, while in the groups with the other routes of administration, values of $3-4 \times 10^3$ were reached. The protection conferred by the intramuscular vaccine was 45 % (9 of 20 animals), compared to the rest of the groups (subcutaneously and with gene gun) that only protected 10 % of the animals⁽⁸⁵⁾.

The potential of a DNA vaccine formulated based on the pTARGET plasmid transformed with the protein esterase cp09720⁽⁸⁶⁾ was compared with a subunit vaccine with recombinant CP09720 adjuvated with aluminum hydroxide. Both were evaluated in BALB/c mice, with the recombinant protein vaccine being the one that induced the highest titer of IgG1 and IgG2 antibodies. The two vaccines were able to increase IFN- γ expression, although the subunit vaccine presented the highest levels of IFN- γ mRNA. Protection levels against the challenge were 58.3 % in animals vaccinated with recombinant esterase, while the pTARGET DNA/protein esterase cp09720 vaccine only protected 16.6 %.

Recombinant protein subunit vaccines

Subunit vaccines combine antigens such as lipopolysaccharides, recombinant proteins, or synthetic peptides. These vaccines are very safe, but not very immunogenic, so adjuvant substances that enhance the response of the immune system are used. The PLD protein obtained by recombinant route has been one of the most used for the development of subunit

vaccines. A group of researchers from the United Kingdom determined the potentialities of a vaccine from 50 µg of PLD obtained recombinantly (PLDr) in *E. coli* and its combination with 1.25×10^{10} cells/ml of whole cultures of *C. pseudotuberculosis* inactivated with formalin. In this work, the control group was vaccinated with the commercial Glanvac 3 vaccine (Commonwealth Serum Laboratories (CSL) Ltd., Victoria, Australia). The highest levels of antibodies were detected in the groups immunized with the PLDr vaccine and the vaccine of PLDr + inactivated whole cells, compared to the control groups⁽⁷³⁾.

The PLDr protein together with whole cultures of *C. pseudotuberculosis* biovar *ovis* and *equi*, inactivated with formalin were used for the immunization of sheep. The detection of anti-PLD antibody levels by ELISA allowed detecting that the vaccinated animals presented an increase in IgG after the second booster dose, but after the challenge, there was a decrease in the OD from 0.65 to 0.55, although the levels remained above the cut-off value for 20 weeks. No lesions were observed in external and internal lymph nodes, compared to the unvaccinated control group where 80 % of the animals presented lesions and manifestations of the disease. Both vaccines were able to protect the animals from the challenge with a virulent strain. In this work for the first time, sheep are immunized with a biovar *equi* strain in combination with PLDr⁽⁸⁷⁾.

Different recombinant proteins rCP09720 (esterase), rCP01850 (L14 protein binding to the 50S rRNA subunit) and PLD (rPLD) have also been evaluated in the immunization of BALB/c mice. In this study, survival rates after challenge with a virulent strain were 30 % (rPLD), 40 % (rPLD + rCP09720) and 50 % (rPLD + rCP01850). The vaccine rPLD + rCP01850 was able to induce a cellular immune response, significantly increasing levels of IFN- γ and TNF- α , while IL4 and IL12 production was not detected⁽⁸⁸⁾.

A live attenuated strain of *Mycobacterium bovis* BCG (Bacillus-Calmette-Guerin) was also used for the expression of recombinant PLD in the pUS2000 plasmid. The system was not efficient for elevated expression of the PLD protein but was effective for vaccination and protection in a mouse model. Immunization of BALB/c mice with 10^6 CFUs of *M. bovis* pUS2000/PLD for PLD expression, as well as with *M. bovis* pUS2000/PLD + 50µg of purified PLDr and the unmodified *M. bovis* strain, induced elevated antibody production compared to the negative control (100 µl of NaCl 0.9 %), but without significant differences between the vaccinated groups. This is because the *M. bovis* strain alone is able to induce an elevated humoral and cellular immune response. However, faced with the challenge with 2×10^4 CFUs of the virulent strain MIC-6, the group vaccinated with *M. bovis* pUS2000/PLD experienced a significant increase in IgG levels compared to the rest of the groups. The cellular immune response was evaluated by measuring the production levels of IFN- γ and IL-10 in the spleen cell culture supernatants of the vaccinated animals, after being stimulated with 8 µg/ml of PLDr. The levels of IFN- γ and IL-10 were higher in the cell culture of the group that received a reactivation of the vaccination with 50 µg of PLDr. The level of

protection conferred by these formulations was 88 % in animals vaccinated with *M bovis* pUS2000/PLD+ 50 µg of PLDr, 77 % for group *M. bovis* pUS2000/PLD and 66 % for the non-modified *M. bovis* group. The protective immune response generated by this whole cell vaccine of *M. bovis* BCG modified to express PLD could cause the activation of several populations of T cells due to the variety of antigens (lipids, proteins and carbohydrates) of the formulation. Then re-immunization with 50 µg of PLD obtained recombinantly stimulates increased proliferation of T cells specific to this particular antigen⁽⁸⁹⁾.

Subunit vaccines have also been developed using the recombinant CP40 protein. The preparation of PLD vaccines from *C. pseudotuberculosis* culture supernatants usually contain other antigens which could be contributing to the protective immune response. The CP40 protein was identified in inactivated vaccine preparations through immunoblot assays, where it was observed that sera from animals vaccinated with Glanvac 6 could intermittently recognize this protein, suggesting that it was present in some vaccine batches⁽⁸²⁾. In an experimental study in sheep, immunization with 100 µg of recombinant CP40 protected 82 % of the animals, with a decrease in lung lesions by 98 %. No relationship was found between decreased development of lung lesions and antibody titer, so it was assumed that cellular response, as antibody-dependent cellular cytotoxicity, could be responsible for protection⁽⁴³⁾.

Subsequently, the comparative evaluation of four vaccine formulations was carried out, which used as immunogens the recombinant CP40 protein and the CP09 strain attenuated by induced mutagenesis. The live attenuated strain CP09 of *C. pseudotuberculosis* was not able to induce a humoral immune response in the vaccinated mice, nor challenged with a virulent strain. Animals vaccinated with formulations that included CP40r had a significant increase in the titer of IgG1 antibodies. However, these groups, after the challenge, experienced a significant increase in IgG2 levels, the maximum being reached by animals immunized with CP40r. The formulation based on CP40r protected 90 % of the animals from the challenge with the virulent strain, followed by the vaccinated group with the attenuated strain CP09 + CP40r with 70 %, while vaccination with CP40r followed by re-immunization with CP09 only protected 60 %⁽⁹⁰⁾.

Another group of researchers performed the evaluation in BALB/c mice of a CP40r subunit vaccine with different adjuvants, saponin or Freud's complete adjuvant (FCA). Animals immunized with CP40r/saponin showed elevated values in complete levels of antibodies and IgG2a, IgG2b and IgG3, with statistically significant differences with respect to the control group. The group vaccinated with CP40r/ACF showed significant differences in complete levels of IgG, IgG2a and IgG2b. Both vaccine formulations protected 100 % of the animals challenged with 10⁴ CFUs of the virulent strain C57 of *C. pseudotuberculosis*, with a tendency towards a Th1 response. Reactivity and production of specific isotypes IgG2a, IgG2b and IgG3 are associated with the action of pro-inflammatory cytokines such as IFN-γ

and CD8+ T cells, which activate B cells by modifying the immunoglobulin heavy chain. The use of different adjuvants did not influence the antibody response, so the use of saponin is proposed to replace Freud's adjuvant, which is toxic in sheep⁽⁹¹⁾.

Conclusions

Caseous lymphadenitis continues to be a challenge for sheep and goat producers worldwide. The most recent studies have focused on the identification of new molecules involved in the mechanisms of pathogenicity and virulence of *C. pseudotuberculosis*, for subsequent evaluation as vaccine candidates. To date, encouraging results have been obtained with formulations based on PLD exotoxin or CP40 endoglycosidase, obtained recombinantly. It should be noted that the combination of these molecules has not been evaluated in the same vaccine, which would be a proposal that would favor the activation of the humoral and cellular immune response. On the other hand, the application of computational analysis in reverse vaccinology studies is currently one of the most used tools in the search for vaccine candidate molecules. Undoubtedly, work with the use of these technologies that constitute an efficient alternative for the identification of new virulence factors should continue, as well as the *in-silico* evaluation of molecules with immunogenic potential for the development of effective vaccines.

Conflict of interests

The authors declare that there is no conflict of interests.

Literature cited:

1. Dorella FA, Pacheco LG, Oliveira SC, Miyoshi A, Azevedo V. *Corynebacterium pseudotuberculosis*: microbiology, biochemical properties, pathogenesis and molecular studies of virulence. Vet Res 2006;37(2):201–218.
2. Paton MW, Rose IR, Hart RA, Sutherland SS, Mercy AR, Ellis TM, *et al.* New infection with *Corynebacterium pseudotuberculosis* reduces wool production. Aust Vet J 1994; 71:47-49.

3. Collett MG, Bath GF, Cameron CM. *Corynebacterium pseudotuberculosis* infections. In: Coetzer J, Thomson GR, Justin RC, editores. Infectious diseases of livestock with special reference to Southern Africa. Cape Town, South Africa: Oxford University Press; 1994:1387–1395.
4. Schreuder BE, Ter Laak EA, De Gee AL. *Corynebacterium pseudotuberculosis* in milk of CL affected goats. Vet Rec 1990;127(15):387.
5. Faeza NMN, Jesse FFA, Hambal IU, Odhah MN, Umer M, Wessam MMS, *et al.* Responses of testosterone hormone concentration, semen quality, and its related pro-inflammatory cytokines in bucks following *Corynebacterium pseudotuberculosis* and its mycolic acid infection. Trop Anim Health Prod 2019;51(7):1855-1866.
6. Paton MW, Walker SB, Rose IR, Watt GF. Prevalence of Caseous lymphadenitis and usage of Caseous lymphadenitis vaccines in sheep flocks. Aust Vet J 2003;81:91-95.
7. Windsor PA. Control of Caseous Lymphadenitis. Vet Clin Food Anim 2011;27:193–202.
8. Williamson LH. Caseous lymphadenitis in small ruminants. Vet Clin North Am Food Anim Pract 2001;17(2): 359-371.
9. Aleman MR, Spier SJ. *Corynebacterium pseudotuberculosis* infections. In: Smith PB, editor. Large animal internal medicine, 3rd ed. St. Louis: Mosby Co. 2002:1076–1084.
10. Bastos BL, Dias PRW, Dorella FA, Ribeiro D, Seyffert N. *Corynebacterium pseudotuberculosis*: Immunological responses in animal models and zoonotic potential. J Clin Cell Immunol 2012;S4 (005):1-15.
11. Gao H, Ma Y, Shao Q, Hong Q, Zheng G, Li Z. Genome sequence of *Corynebacterium pseudotuberculosis* strain KM01, isolated from the abscess of a goat in Kunming, China. Genome Announc 2018;6(11):e00013-18.
12. Windsor P. Managing control programs for ovine Caseous lymphadenitis and Paratuberculosis in Australia, and the need for persistent vaccination. Vet Med Auckl 2014;5:11-22.
13. de Farias AEM, Alves JRA, Alves FSF, Pinheiro RR, Faccioli MPY, Lima A MC, *et al.* Seroepidemiological characterization and risk factors associated with seroconversion to *Corynebacterium pseudotuberculosis* in goats from Northeastern Brazil. Trop Anim Health Pro 2019;51(4):745-752.
14. Debien E, Hélie P, Buczinski S, Leboeuf A, Bélanger D, Drolet R. Proportional mortality: A study of 152 goats submitted for necropsy from 13 goat herds in Quebec, with a special focus on Caseous lymphadenitis. Can Vet J 2013;54:581-587.

15. Varela GJA, Montes de Oca JR, Acosta JD, Hernández FL, Morales EV, Monroy SGH. First report of isolation and molecular characterization of the pathogenic *Corynebacterium pseudotuberculosis* from sheep and goats in Mexico. *Microb Pathog* 2018;117:304-309.
16. Parise D, Parise M, Viana MVC, Muñoz BAV, Cortés-Pérez YA, Azevedo V *et al.* First genome sequencing and comparative analyses of *Corynebacterium pseudotuberculosis* strains from Mexico. *Stand in Genomic Sci* 2018;13(21).
17. Robins R. Focus on Caseous lymphadenitis. *State Vet J* 1991;1:7–10.
18. Binns SH, Bailey M, Green LE. Postal survey of ovine Caseous lymphadenitis in the United Kingdom between 1990 and 1999. *Vet Record* 2002;150(9):263-268.
19. Paton M, Rose I, Hart R, Sutherland S, Mercy A, Ellis T. Post-shearing management affects the seroincidence of *Corynebacterium pseudotuberculosis* infection in sheep flocks. *Prev Vet Med* 1996;26(3-4):275-284.
20. Santos LM, Stanisic D, Menezes UJ, Mendonça MA, Barral TD, Seyffert N, *et al.* Biogenic silver nanoparticles as a post-surgical treatment for *Corynebacterium pseudotuberculosis* infection in small ruminants. *Front Microbiol* 2019;10:824.
21. Stanisic D, Fregonesi NL, Barros CHN, Pontes JGM, Fulaz S, Menezes UJ, *et al.* NMR insights on nano silver post-surgical treatment of superficial Caseous lymphadenitis in small ruminants. *RSC Advances* 2018;71.
22. Baird G, Synge B, Dercksen D. Survey of *Caseous lymphadenitis* seroprevalence in British terminal sire sheep breeds. *Vet Record* 2004;154:505-506.
23. Pinto AC, de Sa PHCG, Ramos RTJ, Barbosa S, Barbosa, HPM, Ribeiro AC, *et al.* Differential transcriptional profile of *Corynebacterium pseudotuberculosis* in response to abiotic stresses. *BMC Genomics* 2014;15:14.
24. Gallardo A, Toledo RA, González-Pasayo RA, Azevedo V, Robles C, Paolicchi FA, *et al.* *Corynebacterium pseudotuberculosis* biovar *ovis* evaluación de la sensibilidad antibiótica *in vitro*. *Rev Argent Microbiol* 2019;51(4):334-338.
25. Stefanska I, Gierynska M, Rzewuska M, Binek M. Survival of *Corynebacterium pseudotuberculosis* within macrophages and induction of phagocytes death. *Polish J Vet Sci* 2010;13(1):143-149.
26. OIE, Organización Mundial de Sanidad Animal. Informe del grupo ad hoc de la OIE sobre las enfermedades prioritarias para las cuales las vacunas pueden reducir el uso de agentes antimicrobianos en bovinos, ovejas y cabras. Paris, 2018. <http://www.oie.int> > fileadmin > SST > adhocreports > AHG.

27. de Pinho RB, de Oliveira Silva MT, Bezerra FSB. Vaccines for caseous lymphadenitis: up-to-date and forward-looking strategies. *Appl Microbiol Biotechnol* 2021;105:2287–2296.
28. Wattam ARJ, Davis J, Assaf R, Brettin T, Bun C, Conrad N, *et al.* Improvements to PATRIC, the all-bacterial bioinformatics database and analysis resource. *Nucleic Acids Res* 2017;45:535-542.
29. Ruiz JC, D’Afonseca V, Silva A, Ali A, Pinto AC. Evidence for reductive genome evolution and lateral acquisition of virulence functions in two *Corynebacterium pseudotuberculosis* strains. *PLoS One* 2011;6(4):e18551.
30. Burkovski A. The role of corynomycolic acids in *Corynebacterium*-host interaction. *Antonie van Leeuwenhoek* 2018;111(5):717-725.
31. Burkovski A. Cell Envelope of *Corynebacteria*: Structure and Influence on Pathogenicity. *ISRN Microbiol* 2013;935736:11.
32. Muller B, de Klerk LLM, Henton MM, Lane E, Parsons S, Kotze A, *et al.* Mixed infections of *Corynebacterium pseudotuberculosis* and non-tuberculous mycobacteria in South African antelopes presenting with tuberculosis-like lesions. *Vet Microbiol* 2011;147:340–345.
33. Silva A, Schneider MPC, Cerdeira L, Barbosa MS, Ramos RTJ. Complete genome sequence of *Corynebacterium pseudotuberculosis* I19, a strain isolated from a cow in Israel with bovine mastitis. *J Bacteriol* 2011;193:323-324.
34. Sprake P, Gold JR. *Corynebacterium pseudotuberculosis* liver abscess in a mature alpaca (*Lama pacos*). *Can Vet J* 2012;53:387-390.
35. Lopes T, Silva A, Thiago R, Carneiro A, Dorella FA. Complete genome sequence of *Corynebacterium pseudotuberculosis* Strain Cp267, isolated from a Llama. *J Bacteriol* 2012;194:3567-3568.
36. Colom CA, Velarde R, Salinas J, Borge C, Garca BI, Serrano E, *et al.* Management of a Caseous lymphadenitis outbreak in a new Iberian ibex (*Capra pyrenaica*) stock reservoir. *Acta Vet Scand* 2014;56:83.
37. Oliveira M, Barroco C, Mottola C, Santos R, Lemsaddek A, Tavares L, Semedo LT. First report of *Corynebacterium pseudotuberculosis* from Caseous lymphadenitis lesions in Black Alentejano pig (*Sus scrofa domesticus*). *Vet Res* 2014;10:218.
38. Muñoz BAV, Cortés PYA, Arellano RB, Hernández GM, Hernández CR, Díaz AE. Identification of *Corynebacterium pseudotuberculosis* isolated from muscular abscesses in two horses: first report in Mexico. *Equine Vet Educ* 2016;29(8):431-435.

39. Borham M, Oreiby A, El GA, Al GM. Caseous Lymphadenitis in Sudanese and Somalian camels imported for meat consumption in Egypt. *AJVS* 2017;55(2):52-59.
40. Viana MVC, Figueiredo H, Ramos R, Guimares LC, Dorella FA, Azevedo V. Comparative genomic analysis between *Corynebacterium pseudotuberculosis* strains isolated from buffalo. *PLoS ONE* 2017;12(4):e0176347.
41. Soares SC, Silva A, Trost E, Blom J, Ramos R, Carneiro A. The pan-genome of the animal pathogen *Corynebacterium pseudotuberculosis* reveals differences in genome plasticity between the biovar ovis and equi strains. *PLoS ONE* 2013;8(1):e53818.
42. Odhah MN, Jesse FFA, Lawan A, Idris UH, Marza AD, Mahmood ZK, *et al.* Responses of haptoglobin and serum amyloid A in goats inoculated intradermally with *C. pseudotuberculosis* and mycolic acid extract immunogen. *Microb Pathog* 2018;117:243-246.
43. Hodgson ALM, Bird P, Nisbett IT. Cloning, nucleotide sequence, and expression in *Escherichia coli* of the phospholipase D gene from *Corynebacterium pseudotuberculosis*. *J Bacteriol* 1990;172:1256–1261.
44. Kolesnikov YS, Nokhrina KP, Kretynin SV, Volotovskii ID, Martinec J, *et al.* Molecular structure of phospholipase D and regulatory mechanisms of its activity in plant and animal cells. *Biochem Biokhimiia* 2012;77:1-14.
45. Dias-Lopes C, Neshich IAP, Neshich G, Ortega JM, Granier C, Chávez-Olortegui C, *et al.* Identification of new Sphingomyelinases D in pathogenic fungi and other pathogenic organisms. *PLoS ONE* 2013;8(11):e79240.
46. Baird GJ, Fontaine MC. *Corynebacterium pseudotuberculosis* and its role in ovine Caseous Lymphadenitis. *J Comp Pathol* 2007;137(4):179-210.
47. Walker J, Wilson MJ, Brandon MR. Molecular and biochemical characterization of a protective 40-kilodalton antigen from *Corynebacterium pseudotuberculosis*. *Infect Immun* 1995;63:206-211.
48. Shadnezhad A, Naegeli A, Collin M. CP40 from *Corynebacterium pseudotuberculosis* is a endo B-N- acetylglucosaminidase. *BMC Microbiol* 2016;63(1):206-211.
49. Pacheco LGC, Slade SE, Seyffert N, Santos AR, Castro TLP. A combined approach for comparative exoproteome analysis of *Corynebacterium pseudotuberculosis*. *BMC Microbiol* 2011;11:12.

50. Paule BJ, Meyer R, Moura-Costa LF, Bahia RC, Carminati R, Regis LF. Three-phase partitioning as an efficient method for extraction / concentration of immunoreactive excreted-secreted proteins of *Corynebacterium pseudotuberculosis*. *Protein Expr Purif* 2004;34:311-316.
51. Silva WM, Dorella FA, Soares SC, Souza GHM, Seyffert N, Azevedo V, *et al.* A shift in the virulence potential of *Corynebacterium pseudotuberculosis* biovar *ovis* after passage in a murine host demonstrated through comparative proteomics. *BMC Microbiol* 2017;17(1):55.
52. Corrêa JI, Stocker A, Castro ST, Vale V, Brito T, Bastos B, *et al.* *In vivo* and *in vitro* expression of five genes involved in *Corynebacterium pseudotuberculosis* virulence. *AMB Expr* 2018;8:89.
53. Ibraim IC, Parise MT, Tadra MZ, de Paula TL, Wattam AR, Azevedo V, *et al.* Transcriptome profile of *Corynebacterium pseudotuberculosis* in response to iron limitation. *BMC Genomics* 2019;20:663.
54. Ibraim IC, Parise MT, Tadra MZ, de Paula TL, Wattam AR, Azevedo V, *et al.* Transcriptome profile of *Corynebacterium pseudotuberculosis* in response to iron limitation. *BMC Genomics* 2019;20:663.
55. Said-Salim B, Mostowy S, Kristof AS, Behr MA. Mutations in *Mycobacterium tuberculosis* RV0444c, the gene encoding anti-sigK, explain high level expression of mpb70 and mpb83 in *Mycobacterium bovis*. *Mol Microbiol* 2006;62:1251–1263.
56. Pacheco LGC, Castro TLP, Carvalho RD, Moraes PM, Dorella FA, Azevedo V, *et al.* A role for sigma factor σ in *Corynebacterium pseudotuberculosis* resistance to nitric oxide/peroxid stress. *Front Microbiol* 2012;3:126.
57. Trost E, Ott L, Schneider J, Schröder J, Jaenicke S, Goesmann A, Husemann P, *et al.* The complete genome sequence of *Corynebacterium pseudotuberculosis* FRC41 isolated from a 12-year-old girl with necrotizing lymphadenitis reveals insights into gene regulatory networks contributing to virulence. *BMC Genomics* 2010;11:728.
58. Costa PM, McCulloch JA, Almeida SS, Dorella FA, Fonseca CT, *et al.* Molecular characterization of the *Corynebacterium pseudotuberculosis* hsp60-hsp10 operon, and evaluation of the immune response and protective efficacy induced by hsp60 DNA vaccination in mice. *BMC Res Notes* 2011;4:243.
59. Pinto GAC, Gomes SP, Queiroz AL, Sousa T, Rodrigues L, Azevedo V, *et al.* Heat shock stress: Profile of differential expression in *Corynebacterium pseudotuberculosis* biovar *Equi*. *Gene* 2018;645:124–130.

60. Silva WM, Seyffert SN, Santos AV, Castro TLP, Pacheco LGC, Azevedo V, *et al.* Identification of 11 new exoproteins in *Corynebacterium pseudotuberculosis* by comparative analysis of the exoproteoma. *Microb Pathog* 2013;1e6:1-6.
61. Al-Gaabary MH, Osman SA, Oreiby AF. Caseous lymphadenitis in sheep and goats: Clinical, epidemiological and preventive studies. *Small Ruminant Res* 2009;87:116–121.
62. Jesse FFA, Odhah MN, Abbad Y, Garba B, Mahmood Z, Hambali IU, *et al.* Responses of female reproductive hormones and histopathology in the reproductive organs and associated lymph nodes of Boer does challenged with *Corynebacterium pseudotuberculosis* and its immunogenic corynomycolic acid Extract. *Microb Pathog* 2020;139:103852.
63. Odhah MN, Jesse FFA, Teik CEL, Mahmood Z, Wahid HA, Mohd LMA, *et al.* Clinico-pathological responses and PCR detection of *Corynebacterium pseudotuberculosis* and its immunogenic mycolic acid extract in the vital organs of goats. *Microb Pathog* 2019;135:103628.
64. Mahmood ZKH, Jesse FF, Saharee AA, Jasni S, Yusoff R, Wahid H. Clinio-pathological changes in goats challenged with *Corynebacterium pseudotuberculosis* and its exotoxin (PLD). *Am J Anim Vet Sci* 2015;10 (3):112.132.
65. Valdivia J. Vida intracelular de *Corynebacterium pseudotuberculosis* [tesis Doctorado]. España, Islas Canarias: Universidad de las Palmas de Gran Canaria. Instituto Universitario de Sanidad animal y Seguridad alimentaria; 2015.
66. Oliveira A, Oliveira LC, Aburjaile F, Benevides L, Tiwari S, Azevedo V, *et al.* Insight of Genus *Corynebacterium*: Ascertaining the role of pathogenic and non-pathogenic species. *Front Microbiol* 2017;8:1937.
67. Oliveira A, Teixeira P, Barh D, Barh D, Ghosh P, Azevedo V. Key amino acids in understanding evolutionary characterization of Mn/Fe-Superoxide dismutase: A phylogenetic and structural analysis of proteins from *Corynebacterium* and Hosts. *Trends Artif Intell* 2017;1(1):1-11.
68. Hard GC. Examination by electron microscopy of interaction between peritoneal phagocytes and *Corynebacterium ovis*. *J Med Microbiol* 1972;5:483-491.
69. Tashjian JJ, Campbell SG. Interaction between caprine macrophages and *Corynebacterium pseudotuberculosis*: an electron microscopy study. *Am J Vet Res* 1983;44:690-693.

70. Paule BJA, Azevedo V, Regis LF, Carminati R, Bahia R. Experimental *Corynebacterium pseudotuberculosis* primary infection in goats: kinetics of IgG and interferon- γ production, IgG avidity and antigen recognition by Western blotting. *Vet Immunol Immunopathol* 2003;96:129-139.
71. Seyffert N, Silva RF, Jardim J, Silva WM, Castro TL, Tartaglia NR, *et al.* Serological proteome analysis of *Corynebacterium pseudotuberculosis* isolated from different hosts reveals novel candidates for prophylactics to control Caseous lymphadenitis. *Vet Microbiol* 2014;174:255-260.
72. Rebouças MF, Portela RW, Lima DD, Loureiro D, Bastos BL. *Corynebacterium pseudotuberculosis* secreted antigen-induced specific gamma-interferon production by peripheral blood leukocytes: Potential diagnostic marker for Caseous Lymphadenitis in sheep and goats. *J Vet Diag Invest* 2011;23:213-220.
73. Fontaine MC, Baird G, Connor KM, Rudge K, Sales J, Donachie, W. Vaccination confers significant protection of sheep against infection with a virulent United Kingdom strain of *Corynebacterium pseudotuberculosis*. *Vaccine* 2006;24:5986–5996.
74. Stanford K, Brogden KA, McClelland LA, Kozub GC, Audibert F. The incidence of Caseous lymphadenitis in Alberta sheep and assessment of impact by vaccination with commercial and experimental vaccines. *Can J Vet Res* 1998;62:38-43.
75. Zoetis, 2020. Zoetis Spain, S.L. Avda. de Europa 20B, Parque Empresarial La Moral. https://www.zoetis.es/_locale-assets/spc/biodyl.pdf. Consultado 16 Abr, 2020.
76. Medrano G, Hung ACh, Alvarado AS, Li EO. Evaluación de una vacuna contra *Corynebacterium pseudotuberculosis* en ratones albinos. *Rev Inv Vet* 2003;14(1):61-67.
77. Braga WU. Protection in alpacas against *Corynebacterium pseudotuberculosis* using different bacterial components. *Vet Microbiol* 2007;119:297–303.
78. Eggleton DG, Doidge CV, Middleton HD, Minty DW. Immunisation against ovine caseous lymphadenitis: efficacy of monocomponent *Corynebacterium pseudotuberculosis* toxoid vaccine and combined clostridial-corynebacterial vaccines. *Aust Vet J* 1991;68:320421.
79. Syame SM, Abuelnaga ASM, Ibrahim ES, Hakim AS. Evaluation of specific and non-specific immune response of four vaccines for caseous lymphadenitis in sheep challenged. *Vet World* 2018;11(9):1272-1276.

80. Moura-Costa LF, Bahia RC, Carminati R, Vale VL, Paule BJ, *et al.* Evaluation of the humoral and cellular immune response to different antigens of *Corynebacterium pseudotuberculosis* in Canindé goats and their potential protection against Caseous lymphadenitis. *Vet Immunol Immunopathol* 2008;126:131-141.
81. Hodgson ALM, Krywult J, Corner LA, Rothel JS, Radford AJ. Rational attenuation of *Corynebacterium pseudotuberculosis*: Potential cheesy gland vaccine and live delivery vehicle. *Infect Immun* 1992;60(7):2900-2905.
82. Hodgson ALM, Tachedjian M, Corner LA, Radford AJ. Protection of sheep against Caseous lymphadenitis by use of a single oral dose of live recombinant *Corynebacterium pseudotuberculosis*. *Infect and Immunol* 1994;62(12):5275-5280.
83. Ribeiro D, Rocha FS, Leite KM, Soares SC, Silva A, Portela RW, *et al.* An iron-acquisition-deficient mutant of *Corynebacterium pseudotuberculosis* efficiently protects mice against challenge. *Vet Res* 2014;45:28.
84. Chaplin PJ, De Rose R, Boyle JS, McWaters P, Kelly J, Tennent JM, *et al.* Targeting improves the efficacy of a DNA vaccine against *Corynebacterium pseudotuberculosis* in sheep. *Infect Immun* 1999;67:6434–6438.
85. De Rose R, Tennent J, McWaters P, Chaplin PJ, Wood PR, Kimpton W, *et al.* Efficacy of DNA vaccination by different routes of immunisation in sheep. *Vet Immunol Immunopathol* 2002;90:55-63.
86. Brum AA, Silva AFR, Silvestre FB, Collares T, Begnine K, Kommling F, *et al.* Recombinant esterase from *Corynebacterium pseudotuberculosis* in DNA and subunit recombinant vaccines partially protects mice against challenge. *J Med Microb* 2017;66:635-642.
87. Moussa IM, Mohamed SA, Ashgan M, Hessain SA, Kabli E, Hassan AH, *et al.* Vaccination against *Corynebacterium pseudotuberculosis* infections controlling Caseous lymphadenitis (CLA) and oedematousskin disease. *Saudi J Biol Sci* 2016;23:718–723.
88. Silva MTO, Bezerra FSB, de Pinho RB, Begnini KR, Seixas FK, Collares T, *et al.* Association of *Corynebacterium pseudotuberculosis* recombinant proteins rCP09720 or rCP01850 with rPLD as immunogens in Caseous lymphadenitis immunoprophylaxis. *Vaccine* 2018;36(1):74-83.
89. Leal KS, Silva TO, Silva AFR, Brilhante FSB, Begnini K, Seixas F, *et al.* Recombinant *M. bovis* BCG expressing the PLD protein promotes survival in mice challenged with a *C. pseudotuberculosis* virulent strain. *Vaccine* 2018;36:3578–3583.

90. Silva JW, Droppa-Almeida D, Borsuk S, Azevedo V, Portela RW. *Corynebacterium pseudotuberculosis* cp09 mutant and cp40 recombinant protein partially protect mice against caseous lymphadenitis. *BMC Vet Res* 2014;10:965.
91. Droppa-Almeida D, Vivas WL, Silva KK, Rezende AF, Simionatto S. Recombinant CP40 from *Corynebacterium pseudotuberculosis* confers protection in mice after challenge with a virulent strain. *Vaccine* 2016;34(8):1091–1096.