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MINI-REVIEW

Vaccines for caseous lymphadenitis: up-to-date and forward-looking strategies

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Abstract

Caseous lymphadenitis (CLA) is an infectious chronic disease responsible for economic losses in sheep and goat breeding worldwide. CLA has no effective treatment, evidencing the vaccination schedule as the best control strategy. Although some commercial vaccines have been available, none of them provides total protection, which is sometimes insufficient and does not reach the same efficiency when compared in sheep and goats. They also have questionable safety levels and side effects. In light of this, several experimental vaccines are in development in order to improve safety, reproducibility, and protective immune response against the etiologic agent of CLA, *Corynebacterium pseudotuberculosis*. In this review, we discussed aspects as antigen, adjuvant, routes of administration, protection level, and animal models used in CLA vaccine development, as well the challenges and future perspectives.

Key points

- *Caseous lymphadenitis (CLA) does not have an appropriate commercial vaccine.*
- *Different experimental vaccines are in development aiming to protect against Corynebacterium pseudotuberculosis.*
- *An ideal vaccine for CLA is necessary for the disease control.*

Keywords *Corynebacterium pseudotuberculosis* · Vaccine development · Adjuvant · Small ruminant · Immunoprophylaxis

Introduction

Globally, caseous lymphadenitis (CLA) is one of the most common and important infections caused by corynebacterias, resulting in significant losses in countries where small ruminant production is substantial (Smith and Sherman 2009). The main challenges for the immunoprophylaxis of CLA are the vaccine efficacy and its correct use in association to sanitary management by the farmers. In most countries, the control of CLA depends on vaccination; however, the disease persists even after a prolonged vaccination period, as reported in Australia (Windsor 2014). Nowadays, herd vaccination does not prevent the disease, but reduces the number of animals that develop lung lesions, and as older animals are eliminated, infection rates are reduced (Windsor and Bush 2016). In addition, adequate immunization reduces the parasitic burden in the environment when an abscess rupture occurs (Windsor 2011).

Corynebacterium pseudotuberculosis, the etiological agent of CLA, is a facultative intracellular bacterium. Therefore, the ideal vaccine should induce both cellular and humoral immunity (Bastos et al. 2012). The commercial CLA vaccine technology available is based on traditional vaccinology, such as toxoids, bacterin, and live attenuated bacteria (Eggleton et al. 1991; Piontkowski and Shivvers 1998; Meyer et al. 2002). Only in the last few years, modern strategies started to be tested in experimental level aiming to increase efficacy and safety of CLA vaccines. So, subunit recombinant, DNA, and vectored vaccines have been used in an attempt to induce proper immunity and high protection (Brum et al. 2017; Leal et al. 2018; Rezende et al. 2020). Most of the researchers have been using IgG and IFN-γ as immunological markers for good quality CLA vaccines (Bastos et al. 2012; Thais et al.

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2018). However, a limited number of adjuvants have been tested, and new studies using various antigen-adjuvant combinations are needed.

This review presents a panorama of the main commercial and experimental vaccines already developed for CLA immunoprophylaxis and provides some discussion on the main challenges and key points for future research and development.

Commercial CLA vaccines

Commercial CLA vaccines are available and licensed in some countries. Table 1 shows the main commercially available vaccines against CLA to date. Most of these commercial vaccines are multipurpose toxoid vaccines formulated with inactivated PLD of *C. pseudotuberculosis* associated with antigens from pathogens of the genus *Clostridium* (*Clostridium tetani*, *Clostridium perfringens*, *Clostridium novyi*, *Clostridium chauvoei*, and *Clostridium septicum*) (Dorella et al. 2006). Glanvac® (Vetrepharm Inc, England) and Biodectin® (Fort Dodge LTD, Australia) are examples of such vaccine formulations.

Glanvac® is a vaccine developed in Australia whose preparation consists in formalin-inactivated exotoxin plus incomplete Freud's adjuvant (Smith and Sherman 2009). Glanvac® was the first commercial vaccine available to the market in 1984 (Windsor 2014). Currently, 9 types of Glanvac vaccines are commercially accessible (Table 1) (Faccioli-Martins et al. 2014). This vaccine formulation using *Clostridium* antigens has been shown to be convenient to use, relatively inexpensive and easy to market. However, optimal protection requires the use of multiple doses per lamb and an annual booster administered to adult sheep (Jorge et al. 2016).

Biodectin® is a sheep product consisting of a fixed combination of six antigenic fractions (4 toxoids and two formalin-treated bacterial cultures) and one endectocide (moxidectin) (MSSSI, 2019). That vaccine is produced by Fort Dodge Animal Health (now Pfizer, www.pfizer.com.br) and commercially available in many countries (Bastos et al. 2012).

Caseous D-T® (Colorado Serum Co., USA) is based on the combination of a bacterin and toxoid of *C. pseudotuberculosis* and two clostridial toxoids (*Cl. perfringens* type D, *Cl. tetani*) (Baird and Fontaine 2007). Caseous D-T® presents two formulations: a composition containing only clostridial toxoids (one component), and another containing a combination of *C. pseudotuberculosis* bacteria and toxoids (two components) (Dorella et al. 2009). The use of Caseous D-T® formulated

Table 1 Commercial caseous lymphadenitis vaccines

Commercial name	Developer	Licensed use/country	Composition
Glanvac® 3	Zoetis	Sheep and goat/South Africa	*CP* toxoid associated with two clostridial toxoids
Glanvac® 6	Zoetis	Sheep/New Zealand	*CP* toxoid associated with five clostridial toxoids and bacterins
Glanvac® 3 B12	Zoetis	Sheep/Australia	*CP* toxoid associated with two clostridial toxoids + B12 vitamin
Glanvac® 3 Vaccine (for goats)	Zoetis	Sheep and goat/Australia	*CP* toxoid associated with two clostridial toxoids
Glanvac® 3S B12	Zoetis	Sheep/Australia	*CP* toxoid associated with two clostridial toxoids + selenium + B12 vitamin
Glanvac® 6 (for goats)	Zoetis	Sheep and goat/Australia	*CP* toxoid associated with five clostridial toxoids and bacterins
Glanvac® 6S	Zoetis	Sheep/Australia	*CP* toxoid associated with two clostridial toxoids and bacterins + selenium
Glanvac® 6 B12	Zoetis	Sheep/Australia	*CP* toxoid associated with five clostridial toxoids + B12 vitamin
Glanvac® 6	Zoetis	Sheep and goat/Brazil	*CP* toxoid associated with five clostridial toxoids and bacterins
Vacina 1002®	Labovet Produtos Veterinários	Sheep and goat/Brazil	Live attenuated *CP*
Caseous D-T®	Colorado Serum Co.	Sheep/EUA, Canada	Combination of three antigenic substances, *Clostridium perfringens* type D; *Cl. tetani* and *CP*
Linfovac®	Vencofarma Lab.	Sheep and goat/Brazil	Live attenuated *CP* suspension
Case-Bac®	Colorado Serum Co.	Sheep/EUA, Canada	Detoxified and purified whole culture of *CP*
Biodectin®	Fort Dodge LTD.	Sheep/many countries	*CP* toxoid associated with *Clostridium* antigens + endectocide
CL Bacterin Vaccine	Texas Vet Labs.	Goat/EUA	*CP* bacterin

The table shows the commercial name of the vaccine, the developer company, the countries where the vaccine is commercially licensed, and the formulation

CP Corynebacterium pseudotuberculosis

Table 2 Experimental vaccines developed against caseous lymphadenitis

Immunogen	Adjuvant	Route	% protection*	Animal model	Author
CP bacterin	A.P	–	–	Sheep	(Cameron et al. 1972)
CP bacterin or sonicated *CP* wall	W.I.O	I.M	–	Sheep	(Brogden et al. 1984)
CP bacterin	M.D	I.P	30	Mice	(Brogden et al. 1985)
	T.D		20		
	F.C		40		
	W.I.O		22		
	BCG		10		
	Heat-killed *Cryptosporidium parvum*		20		
Sonicated *CP* wall	M.D	I.P	80	Mice	(Brogden et al. 1985)
	T.D		44		
	F.C		40		
	W.I.O		80		
	BCG		11		
	Heat-killed *C. parvum*		20		
Formalized exotoxin of *CP*	F.I	S.C	50	Goat	(Brown et al. 1986)
CP bacterin	–	S.C	–	Sheep	(LeaMaster et al. 1987)
CP bacterin	A.P	–	–	Goat	(Ribeiro et al. 1988)
CP bacterin and toxoid	Levamisole	–	–	Goat	(Holstad et al. 1989)
CP bacterin and toxoid	–				
CP bacterin and toxoid	–	–	–	Goat	(Holstad 1989)
CP bacterin + M.D	W.I.O	–	–	Sheep	(Brogden et al. 1990)
CP bacterin + M.D				Mice	
CP bacterin				Sheep	
CP bacterin				Mice	
Dried whole *CP* cells	M.O + Arlacel A	I.M	–	Sheep	(Menzies et al. 1991)
Dried whole *CP* cells				Goat	
Filtrated culture supernatant exotoxins of *CP*	Block polymer	–	–	Sheep	(Ellis et al. 1991)
Cell-free toxoid	A.H	S.C	–	Sheep	(Eggleton et al. 1991c)
Toxoid + *CP* bacterin					
Varying concentrations of *CP* toxoid + 5 clostridial toxoids	A.H	S.C	–	Sheep	(Eggleton et al. 1991b)
Live attenuated 1002 *CP* strain		I.D	–	Goat	(Ribeiro et al. 1991)
CP bacterin	A.P				
PLD-negative *CP* strain (Toxminus)	–	S.C	–	Sheep	(Hodgson et al. 1992)
PLD-negative *CP* strain (Toxminus)	–	O	–	Sheep	(Hodgson et al. 1994)
Recombinant Toxminus					
40 kDa antigen	A.H	S.C	82	Sheep	(Walker et al. 1994)
CP bacterin	M.D + M.O	I.M	–	Sheep	(Brogden et al. 1996)
				Goat	
Live Cp *aroQ* mutant	–	I.P	–	Mice	(Simmons et al. 1997)
Live TB521 *pld* mutant					
Live Cp *aroQ* mutant	–	S.C	–	Sheep	(Simmons et al. 1998)
Live TB 521 *pld* mutant					
Genetically inactivated PLD	–	S.C	44	Sheep	(Hodgson et al. 1999)
Plasmid DNA boCTLA-4-hIg-ΔPLD	–	I.M	70	Sheep	(Chaplin et al. 1999)
Plasmid DNA ΔPLD			56		
Sonicated *CP* cell wall protocol	–	–	–	Goat	(Kutschke et al. 2000)
Lyophilized live attenuated *CP* 1002 strain	–	I.D	–	Goat	(Meyer et al. 2002)
CP protein precipitate	A.H	S.C	60	Mice	Gallardo et al. 2003)

Table 2 (continued)

Immunogen	Adjuvant	Route	% protection*	Animal model	Author
CP bacterin Toxoid	M.O	S.C	– –	Mice	(El-Enbaawy et al. 2005)
CP bacterin + toxoid					
Purified rPLD from *CP* 3/99-5 strain Purified rPLD + *CP* whole cell	A.H	S.C	– 100	Sheep	(Fontaine et al. 2006)
Live *CP*	–	S.C	–	Alpacas	(Braga et al. 2007)
Sonicated *CP* cell wall Filtrated culture supernatant exotoxins	M.D	S.C	–	Alpacas	(Braga 2007)
Purified recombinant mutated PLD *CP* whole cell	M.O	S.C	–	Mice	(Ibrahim et al. 2007)
CP bacterin + toxoid					
Live *CP*					
CP T1 strain culture supernatant Concentrated *CP* T1 strain culture supernatant	F.I CpG	S.C	–	Goat	(Moura-costa et al. 2008)
rHsp60	F.C + F.I	S.C	0	Mice	(Pinho et al. 2009)
CP bacterin *CP* bacterin + mrPLD	M.O	S.C	80 100	Sheep	(Selim et al. 2010)
mrPLD + gamma irradiated *CP*			72		
mrPLD	BCG		66		
pVAX1/*hsp60*	–	I.M	0	Mice	(Costa et al. 2011)
rCP40 CP09 recombinant live strain	SAP –	I.P S.C	90 50	Mice	(Silva et al. 2014)
rCP40 + CP09 recombinant live strain	SAP	I.P/S.C	70		
CZ171053 mutant strain	–	I.P	80	Mice	(Ribeiro et al. 2014)
rCP40	SAP F.C	S.C	100	Mice	(Droppa-almeida et al. 2016)
CP T1 strain	–	I.P	–	Mice	(Lúcia et al. 2016)
rCP09720 pTARGET/*cp09720* DNA vaccine	A.H –	S.C I.M	58.3 16.6	Mice	(Brum et al. 2017)
rPLD rPLD + rCP01850	SAP	S.C	30 50	Mice	(Silva et al. 2018)
rPLD + rCP09720			40		
M. bovis BCG expressing *pld* *M. bovis* BCG expressing *pld* + rPLD boost	–	I.P	77 88	Mice	(Leal et al. 2018)
rCP01850 pTARGET/*cp01850*	A.H –	I.M	0	Mice	(Rezende et al. 2020)
pTARGET/*cp01850* + rCP01850 boost	A.H				
rCP01850	BRPHE	S.C	70	Mice	(Bezerra et al. 2020)

The table shows the vaccinal formulation, route of administration, protection rate, and animal model used in each experiment

CP, *Corynebacterium pseudotuberculosis*; *S.C*, subcutaneous; *I.P*, intraperitoneal; *I.M*, intramuscular; *I.D*, intradermal; *O*, oral; *A.H*, aluminum hydroxide; *A.P*, aluminum phosphate; *W.I.O*, water-in-oil emulsion; *M.O*, mineral oil; *SAP*, saponin; *M.D*, muramyl dipeptide; *F.C*, Freund's complete; *F.I*, Freund's incomplete; *BCG*, bacillus Calmette–Guérin; *T.D*, trehalose dimycolate; *BRPHE*, Brazilian red propolis hydroalcoholic extract

*The % protection depends on the animal model used in the study. When mice are used, the % protection is related to survival, whereas when using sheep or goat, the % protection is due to sterilizing immunity (presence or absence of abscesses)

with bacterin-toxoid in sheep promotes significantly less external, internal, and total abscesses than in control sheep, and high antibody response against both vaccinal components (Piontkowski and Shivvers 1998).

Case-Bac® (Colorado Serum Co., USA) uses the combination of *C. pseudotuberculosis* toxoid and bacterin, but without clostridial toxoids. The use of combined toxoid vaccines can promote a reduction in the number and size of lung abscesses in

animals as well as in the dissemination of CLA in herd (Paton et al. 1995). On the other hand, studies suggested that the addition of formalin-killed cells of *C. pseudotuberculosis* to the toxoid does not improve the protection level, once the number of lesions found in sheep vaccinated with the isolate toxoid or with bacterin-toxoid association is statistically similar (Eggleton et al. 1991c; Eggleton et al. 1991b).

A live-attenuated vaccine of *C. pseudotuberculosis* strain 1002 (Labovet-Produtos Veterinários, Brazil) was developed by the Bahian Agricultural Development Company and released by the Brazilian Ministry of Agriculture and Supply for production and was released for marketing in Brazil since 2000 (Dorella et al. 2009). Strain 1002 is a naturally attenuated strain isolated from a goat in the year 1971 (Ribeiro et al. 1991). The vaccine is industrially produced in liquid form or lyophilized, should be administered annually by subcutaneous route, and showed a protection rate of 83.3% in experimentally infected goats (Dorella et al. 2006). Another vaccine that uses the live attenuated strain 1002 in their formulation is LinfoVac® (Laboratórios Vencofarma do Brasil Ltda, Brazil), licensed for use in sheep and goats, also currently available in Brazil (Bastos et al. 2012).

Although these commercial vaccines have been available for some decades, none of them provides total protection against CLA. The protection obtained is partial and sometimes insufficient and does not all reach the same efficiency when compared in sheep and goats (Williamson 2001). They also have questionable safety levels, presenting side effects such as injury or abscess at injection site, fever, lethargy, and reduced milk production (Stanford et al. 1998; Alves et al. 2007; Ribeiro et al. 2014).

Experimental CLA vaccines

Table 2 compiles data from publications addressing the experimental CLA vaccines published so far. Data are summarized according to antigen, adjuvant, route of administration, protection efficacy, and animal model used in each study.

It is important to mention that the cost per protected animal will be determined by several factors like the cost of a dose, the length of time over which it is protective, the number of doses needed to confer protection and the possibility of side effects (McLeod and Rushton 2007). Because of the globally increasing qualitative and quantitative demands for livestock and their products, vaccine producers are being required to fulfill a set of prescribed specifications, and because of that, the experimental vaccines rely on the advancement of biotechnology. Recombinant subunit and DNA vaccines are currently cost-effective methods of producing antigens that are free from the exogenous materials that are associated with conventional vaccines (Lubroth et al. 2007).

Different periods in CLA vaccine research and development are remarkable. Primarily, *C. pseudotuberculosis* bacterins or total toxoids were tested with optional adjuvant addition. These strategies were encouraged after studies using ewes and alpacas proving that after primary *C. pseudotuberculosis* infection, the animals acquire long-term immunity against secondary exposures (Pépin et al. 1988; Pépin et al. 1993; Braga et al. 2007). In a second phase, the use of recombinant DNA technology updated the immunization strategies: subunit, DNA, and vectorized vaccines aimed reproducibility, safety, and target immune responses through the use of specific epitopes (Moyle and Toth 2013; Hobernik and Bros 2018; Leal et al. 2018).

Bacterin

Administration of whole cell is one of the most studied methods for vaccination against bacterial infection; the advantages include the adjuvant effect and presentation of many protective and undefined antigens. However, some side effects are reported (Pace et al. 1998). Killed bacteria have been used in different CLA vaccine studies (Cameron et al. 1972; Brogden et al. 1984; Brogden et al. 1985; LeaMaster et al. 1987; Ribeiro et al. 1988; Holstad 1989; Holstad et al. 1989; Brogden et al. 1990; Ribeiro et al. 1991; El-Enbaawy et al. 2005; Ibrahim et al. 2007; Selim et al. 2010), being administered with or without adjuvants. Adjuvants used with bacterin studies include aluminum phosphate (Cameron et al. 1972), water-in-oil emulsion (Brogden et al. 1984), muramyl dipeptide (Brogden et al. 1985), levamisole (Holstad et al. 1989), and mineral oil (El-Enbaawy et al. 2005). Besides that, three animal models were used in CLA bacterin studies: sheep (Cameron et al. 1972; Brogden et al. 1984; LeaMaster et al. 1987; Brogden et al. 1990; Selim et al. 2010), goat (Ribeiro et al. 1988; Holstad et al. 1989; Holstad 1989; Ribeiro et al. 1991), and mice (Brogden et al. 1985; Brogden et al. 1990; El-Enbaawy et al. 2005; Ibrahim et al. 2007). As example, lambs immunized with *C. pseudotuberculosis* bacterin had significant increase in antibody titers and in time for the appearance of external natural abscesses (Brogden et al. 1990). Killed *C. pseudotuberculosis* do not completely prevent the disease, but decrease the number of granulomas in sheep and goat (Brogden et al. 1996). Brogden et al. (1985) demonstrated a range in protection level for bacterin varying from 0 to 80% in mice (Brogden et al. 1985). As conclusion, even with variation in formulations and animal models used, in spite of the partial protection provided by bacterin-based vaccines, a significant reduction in the granulomas number is observed in comparison to unvaccinated animals.

Toxoid

Toxoid vaccines, based on treated exotoxins of *C. pseudotuberculosis*, have also been widely described (Brown et al. 1986; Holstad et al. 1989; Eggleton et al.

1991b; Eggleton et al. 1991a; Ellis et al. 1991; Braga 2007; Moura-costa et al. 2008). This strategy is based on using treated exotoxins with formaldehyde or heat producing toxoids that are being administered in low doses of infection in a first immunization, to provide protection to the vaccinated animal in a subsequent infection (Braga et al. 2007). Therefore, toxoid-based vaccines demonstrated good antibody levels against exotoxins, in addition to numerous cellular antigens by ELISA, which can contribute to decrease the spread of the disease in sheep (Eggleton et al. 1991b, c; Ellis et al. 1991; Paton et al. 1995). This strategy, similar to bacterin-based vaccines achieving only partial protection, triggers some adverse reactions at the inoculation site (Braga 2007), but decreases the prevalence and number of abscesses in the vaccinated animals after *C. pseudotuberculosis* challenge (Piontkowski and Shivvers 1998).

Live attenuated vaccines

Strategies based on live attenuated vaccines are also described (Hodgson et al. 1992; Hodgson et al. 1994; Simmons et al. 1997; Simmons et al. 1998; Meyer et al. 2002; Moura-costa et al. 2008). While some studies describe varying protection levels and stimuli in humoral and cell-mediated immune responses, others showed weak immune response and no protection (Simmons et al. 1998; Moura-costa et al. 2008). In addition, undesirable abscesses at the inoculation site are generally reported when high doses of vaccine are used (Hodgson et al. 1992). Toxminus, a phospholipase D (*pld*)-deleted *C. pseudotuberculosis* strain, resulted in lower toxicity, higher protection, and reduction in the number and intensity of typical CLA granulomas in sheep, compared to unvaccinated control (Hodgson et al. 1992). When Toxminus was modified by the insertion of the genetically inactivated PLD, as live vaccine vector to orally immunize sheep, a 100% protection against the *C. pseudotuberculosis* challenge was reported, showing the potential of that strategy (Hodgson et al. 1994). AroQ mutants, developed by allelic exchange technique in *C. pseudotuberculosis*, were tested in sheep and failed in conferring protection, besides reducing the clinical severity of challenge (Simmons et al. 1998). The use of live attenuated vaccines is inexpensive and the possibility of being used as vaccine vectors represents a promising strategy (Simmons et al. 1997).

Subunit purified vaccines and PLD-based

Different techniques were used to isolate fractions of *C. pseudotuberculosis* proteins, in order to identify antigens with immunodominant and protective characteristics (Braithwaite et al. 1993; Paule et al. 2004) for the prophylaxis of CLA. Phospholipase D (PLD) is the more explored exotoxin in vaccine trials for CLA. PLD is described as a facilitator of the infiltration and dissemination of *C. pseudotuberculosis* in the host, also being considered the most relevant virulence factor of the bacterium (Dorella et al. 2006; McKean et al. 2007). Its action and expression in vivo describe it as the main factor of virulence and probable protective antigen (McKean et al. 2007). As described by Hodgson et al. (1992), PLD-negative *C. pseudotuberculosis* strain (Toxminus) is incapable of inducing caseous lymphadenitis. Therefore, several CLA vaccinal formulations using purified PLD as the major component have already been described (Hodgson et al. 1999; Fontaine et al. 2006; Selim et al. 2010; Thais et al. 2018; Leal et al. 2018).

Walker et al. (1994) described a 40-kDa antigen (CP40) from *C. pseudotuberculosis*, identified by locally derived antibody-secreting cells (ASC) as a vaccinal target. CP40 was able to reduce 82% infection rate and 98% of lung lesions in sheep. In addition, sera from vaccinated sheep exhibited humoral response to CP40, demonstrated in immunoblots, suggesting an important role for the immunity against caseous lymphadenitis (Walker et al. 1994).

Subunit recombinant vaccines

Studies applying subunit recombinant vaccines against CLA have been in evidence in recent years mainly by their safety, related to the use of pure or semi-pure antigens and by advances in bioinformatics approaches that allowed the identification of proteins or glycoproteins with potential protective effect (Doytchinova and Flower 2007; Rezende et al. 2016). To date, several studies have used the subunit recombinant vaccine strategy against caseous lymphadenitis (Pinho et al. 2009; Selim et al. 2010; Silva et al. 2014; Droppa-almeida et al. 2016; Brum et al. 2017; Silva et al. 2018; Leal et al. 2018; Rezende et al. 2020; Bezerra et al. 2020). This strategy uses only a part of the pathogen for immune recognition; however, the high purity level decreases its immunogenicity, requiring adjuvants to improve the immune response (Christensen 2016). Due to this fact, different adjuvants have already been tested in combination to recombinant proteins: Freund's complete adjuvant (Pinho et al. 2009; Droppa-almeida et al. 2016), mineral oil (Selim et al. 2010), saponin (Silva et al. 2014; Droppa-almeida et al. 2016; Silva et al. 2018), aluminum hydroxide (Brum et al. 2017; Rezende et al. 2020), and more recently, propolis (Bezerra et al. 2020).

The Hsp60 protein in a recombinant vaccine was tested in Balb/c mice, increasing the anti-rHsp60 IgG and IFN-gamma levels; however, all mice died after intraperitoneal challenge with *C. pseudotuberculosis* (Pinho et al. 2009). In two other studies, recombinant protein CP40 (rCP40) was capable to promote protection rates of 90 and 100% in mice, but in both studies, the negative control group protected 20% of mice (Silva et al. 2014; Droppa-almeida et al. 2016). The recombinant PLD (rPLD) alone and in association with whole-*C. pseudotuberculosis* cells seems to restrict bacteria's

dissemination after challenge, conferring significant protection against infection in sheep (Fontaine et al. 2006).

Interesting results were obtained with acid phosphatase rCP01850 from *C. pseudotuberculosis*, once when associated to aluminum hydroxide, a mixed Th1/Th2 profile was obtained with no significant protection (Rezende et al. 2020). However, the combination of rCP01850 with hydroalcoholic extract of Brazilian red propolis improved protection to 70% and significant levels of IgG, IFN-gamma, and IL-10 were reported (Bezerra et al. 2020), confirming that testing different adjuvants with the same recombinant protein might generate completely different results.

The association of recombinant proteins has been pointed as alternative to improve protection and immunity. Silva et al. (2018) used the recombinant phospholipase D associated with rCP09720 and rCP01850 proteins expressed in *Escherichia coli* in vaccine formulations using saponin as adjuvant to immunize mice (Silva et al. 2018). Protection rates of 40 and 50%, respectively, were obtained in mice after challenge with the virulent strain MIC-6 of *C. pseudotuberculosis*, in comparison to 30% obtained with the isolated use of rPLD, showing the synergism of rPLD with rCP09720 or rCP01850 (Silva et al. 2018).

DNA vaccines

With the advent of DNA vaccination technology, the efficiency of DNA vaccines against *C. pseudotuberculosis* was analyzed, as well as the targeting of these antigens to antigen presenting cells, aiming an increase on the efficiency and in a long-term immune response (Chaplin et al. 1999; Costa et al. 2011; Brum et al. 2017; Rezende et al. 2020). Even with few studies, some interesting results were obtained on this approach. Genetically detoxified PLD (ΔPLD) was used fused with CTLA-4, promoting a significant increase in the magnitude, speed, and longevity of the antibody response in sheep when compared to DNA coding for ΔPLD alone, also offering partial protection against *C. pseudotuberculosis* challenge, similar to that provided by a formalin-inactivated subunit vaccine (Chaplin et al. 1999). In a murine model, Brum et al. (2017) developed a DNA vaccine coding for the CP09720 protein sequence, identified as a promising vaccine target in a pan-secretome study of strains 1002 and C231 (Rezende et al. 2016). However, the pTARGET/*cp09720* vaccine was not effective in inducing an immune response or significant protection after the challenge with the virulent *C. pseudotuberculosis* strain MIC-6 (Brum et al. 2017). Similarly, the intramuscular injection of pTARGET/*cp01850* was not able to protect mice against *C. pseudotuberculosis* challenge (Rezende et al. 2020). Still, pVAX/*hsp60* failed to provide protection against the challenge in mice, despite the IgG-specific humoral immune response induced (Costa et al. 2011).

Vector-based vaccines

Vectored vaccines expressing heterologous genes proved to be a possibility for CLA prophylaxis. So far, one study using this strategy was conducted. The use of bacillus Calmette–Guérin (BCG) as a recombinant vector vaccine expressing the *pld* gene was evaluated (Leal et al. 2018). BCG is excellent for constructing a recombinant vector-based vaccine due to its various advantages, such as adjuvant proprieties and low cost (Leal et al. 2018). When used to immunize Balb/c mice, the vaccine formulation of recombinant *Mycobacterium bovis* BCG expressing the rPLD protein achieved a 77% survival rate, whereas using the *M. bovis* BCG plus rPLD booster, the survival rate increased to 88%; in addition, significant IFN-gamma production was described in both experimental groups (Leal et al. 2018).

Challenges and future perspectives in CLA vaccine development

While vaccines are essential for CLA control, the success of the vaccination program encompasses the correct use of vaccines and good animal management practices (Bastos et al. 2012). In this context, to find a high-efficacy vaccine against CLA is extremely desirable, but it is noteworthy that changing farmers' attitudes towards animal health and welfare practices is still the major challenge (Young et al. 2015).

Furthermore, commercially available vaccines do not achieve full protection, principally if its use is destined to both sheep and goats (Williamson 2001). They have questionable safety levels, oftentimes presenting abscess formation, fever, lethargy, and reduced milk production (Stanford et al. 1998; Alves et al. 2007), emphasizing the need of investments in research and development in CLA vaccines. However, just a limited number of adjuvants were tested with just few antigens, being the main adjuvants reported by different authors were aluminum salts, water-in-oil emulsions, and more recently, saponins. It is known that a same antigen used with different adjuvants could generate completely different results (Guy 2007), since the adjuvant stimulates innate immune by different pathways and drives the adaptive immune response (Didierlaurent et al. 2017). Therefore, further studies should explore different antigen-adjuvant combinations aiming to achieve an adequate cell-mediated immune response, indispensable for combating *C. pseudotuberculosis*.

The use of novel associated strategies and the search for new adjuvants in studies using the target species is necessary, since preliminary articles in mice have shown good results, highlighting that a limitation found in trials for CLA vaccines is the animal model used. Most of the studies developed in the last 10 years used mice as an animal model for testing new vaccinal formulation against CLA, indicating preliminary

studies. It is important to highlight that the choice of the animal model is critical for the success of the vaccine development (Gerdts et al. 2015). Probably, due to their limited accessibility and high housing cost, the small ruminants were less used in CLA vaccine development. This act compromises the technology advance, once although studies with mice can generate important preliminary conclusions. The immunity in rodents is not the same as in small ruminants and could culminate in different efficacy levels if both models were compared. Thus, more experiments using small ruminants should be performed to effectively promote reliable results, leading to offer effective and safer products for the market.

Lastly, it is important to observe the outcome when mice are used in experiments to assess new vaccinal formulations against CLA. In field conditions, CLA is a chronical and frequently subclinical disease, rarely fatal. However, when mice are used in CLA vaccinal trials, the outcome generated is life or death. Sometimes, the animals died in the first experimental days, probably due to sepsis by enormous CFU number inoculated. Experimental designs also should be reformulated, aiming at a better animal welfare and a more reliable reproduction of the disease on the animal model. Approaches such as endpoint establishment were developed as refinement to avoid animal suffering (Silva et al. 2019), and these best conditions on preliminary mouse studies can provide more specific results to progress for advanced research using the sterilizing immunity analysis in sheep and goats, arriving more efficiently in a safe and effective vaccine.

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Declarations

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