Blackleg in cattle: current understanding and future research needs

Rosangela Estel Ziech[1]* **Leticia Trevisan Gressler[2]**
Joachim Frey[3] **Agueda Castagna de Vargas[1]**

[1]Departamento de Medicina Veterinária Preventiva, Universidade Federal de Santa Maria (UFSM), 97105-900, Santa Maria, RS, Brasil.
E-mail: reziech@gmail.com. *Corresponding author.
[2]Instituto Federal Farroupilha (IFFar), Frederico Westphalen, RS, Brasil.
[3]Institute of Veterinary Bacteriology, Vetsuisse Faculty, University of Bern, Bern, Switzerland.

ABSTRACT: *Blackleg is an endogenous acute infection that principally affects cattle, whose etiologic agent is the anaerobic bacterium Clostridium chauvoei. In recent years, the major virulence factors of C. chauvoei have been discovered and described. However, the pathogenesis of blackleg in cattle, and in particular, the movement of the pathogen from the point of entry to the affected tissues is not yet fully elucidated. Disease control is based on appropriate management and vaccination. This review summarizes the latest research findings that contribute toward the understanding of the disease in cattle, provide a foundation to preventive strategies, and identify future research needs.*
Key words: *Clostridium chauvoei, sudden death, myonecrosis.*

Carbúnculo sintomático: compreensão atual e futuras necessidades de pesquisa

RESUMO: *O carbúnculo sintomático é uma infecção endógena, aguda, que acomete principalmente bovinos, cujo agente etiológico é a bactéria anaeróbica Clostridium chauvoei. Recentemente, os principais fatores de virulência do C. chauvoei foram descobertos e descritos. Contudo, a patogênese do carbúnculo sintomático em bovinos, especialmente a circulação do patógeno desde o ponto de entrada até os tecidos acometidos ainda não está completamente elucidada. O controle desta enfermidade é baseado em medidas adequadas de manejo e vacinação. Esta revisão reúne as mais recentes descobertas que contribuíram para o entendimento da doença em bovinos, fornece embasamento para medidas preventivas e aponta futuras necessidades de pesquisa.*
Palavras-chave: *Clostridium chauvoei, morte súbita, mionecrose.*

INTRODUCTION

Blackleg, also known as black quarter, is a generally fatal form of myonecrosis usually observed in young cattle (USEH et al., 2006b). *Clostridium chauvoei*, the etiologic agent of blackleg, is a gram-positive, motile, histotoxic, and sporulating anaerobic bacterium (QUINN et al., 2011). This infectious disease is acute and globally spread among ruminants, causing significant loss in livestock production (FREY & FALQUET, 2015). Although blackleg vaccination has been carried out since 1930, sporadic outbreaks are still recorded annually worldwide (USEH et al., 2006b).

In 1782, Chabert differentiated between blackleg and anthrax on the basis of symptomatic and pathological features (KRIEK & ODENDAAL, 2004). Although blackleg is one of the oldest known diseases affecting cattle, there are important gaps in the understanding of this disease, especially with respect to its pathogenesis. Focusing on the cattle disease, this article aims to offer an overview of the current knowledge about the etiology, virulence factors, epidemiology, pathogenesis, diagnosis, and prevention of blackleg and to identify areas for further research and development.

Epidemiology and clinical and pathological manifestation

Blackleg is an endemic disease in both developed and developing countries and a well-known cause of financial loss to cattle breeders (USEH et al., 2006b). Most cases of blackleg occur during the warm months, or after soil excavation, or during very high annual rainfall that can expose and activate latent spores. In addition, the disease is enzootic in areas with a history of flooding (USEH et al., 2006a; HUANG et al., 2013).

The type of soil and water permeability might represent an important indicative factor in surveillance programs, which was once associated with an increased risk of blackleg cases in Zambia (HANG'OMBE et al., 2000) and Austria (WOLF et al., 2017).

Cattle aged between 6 to 24 months, in good health, are mostly affected (UZAL, 2012). The clinical signs of the hyperacute form of this disease are usually not observed because of sudden death. The acute form of the disease is often reported with swelling and crepitus of affected muscles (SINGH et al., 1993).

The most commonly reported findings in classical blackleg are acute neutrophil necrotizing myositis that affects the skeletal muscle, and visceral myonecrosis, which is rarely diagnosed, but can affect the heart, sublingual muscles, and diaphragm (ASSIS et al., 2010; CASAGRANDE et al., 2015). Other unusual findings include fibrinous pleuritis, pericarditis, epicarditis (DALY et al., 2009), and severe acute necrotizing enteritis (HARWOOD et al., 2007) as well as the highly uncommon meningoencephalitis (MALONE et al., 1986; SAC, 2016).

Etiology and virulence factors

Blackleg is caused by an anaerobic, highly pathogenic, endospore-forming, gram-positive bacterium called *C. chauvoei*, which produces lemon-shaped endospores and requires an enriched medium for growth (QUINN et al., 2011). The first draft genome sequence of a virulent *C. chauvoei* strain became available in 2013; it consists of 2.8 million base pairs (bp) (FALQUET et al., 2013). Moreover, it contains a cryptic plasmid, about 5.5 kbps in size (FREY & FALQUET, 2015). Recently, the full genome sequences of 20 strains of *C. chauvoei*, isolated from four different continents over a period of 64 years, were determined and analyzed. The results of this study showed that the strains analyzed were highly conserved, which further indicates that the evolution of *C. chauvoei* has reached a dead end (RYCHENER et al. 2017).

The relative small genome of *C. chauvoei*, compared to other *Clostridium* species such as *C. difficile* (4.2 million bp) (SEBAIHIA et al., 2006), reflects its adaptation to a restricted host range (bovine, caprine, and ovine), where *C. chauvoei* is able to replicate and cause disease (FREY & FALQUET, 2015). However, a comparative analysis of the circular genome sequences of the *C. chauvoei* type strain (ATCC 10092) and the field strain 12S0467 isolated in Germany showed novel variations in the regulatory genes, indicating that *C. chauvoei* has specific control of the regulatory events, unlike the genomes of other *Clostridium* species (THOMAS et al., 2017).

C. chauvoei has 69 genes dedicated to the mechanisms involved in sporulation and dormancy (FREY & FALQUET, 2015), which might be considered as virulence factors that enable the pathogen to resist adverse environmental conditions and remain potentially infectious over years (GALPERIN et al., 2012). Recently, it was observed that the genes related to sporulation and germination in *C. chauvoei* are homologous to those in *Clostridia* cluster I group, that includes *C. botulinum, C. haemolyticum, C. novyi, C. perfringens, C. tetani, C. septicum*, and *C. chauvoei* (THOMAS et al., 2017). Furthermore, *C. chauvoei* produces several cellular (somatic and flagellar) (CRICHTON et al., 1990) and soluble antigens associated with virulence.

Cellular antigens

Somatic antigens are associated with the bacterial cell. Such antigens are considered crucial immunogenic components involved in the protection against *C. chauvoei* infection, and therefore, current and old vaccines contained bacterins or were solely composed of bacterins (CHANDLER and GULASEKHARAM, 1974). In this sense, some studies aiming to improve vaccine quality have described important characteristics of somatic antigen expression. The amount of somatic antigens varies with bacterial growth as well as environmental conditions such as pH and carbohydrate availability, showing increased expression during the stationary phase of growth in axenic culture medium (CORTINAS et al., 1994). However, the expression of these antigens does not seem to vary among strains from different origins (MATTAR et al., 2002), which underlines the very high genetic similarity observed among strains from all over the globe (RYCHNER et al., 2017).

Flagellar antigens have been studied extensively, highlighting flagellin, which is encoded by the *fliC* gene. Flagellin has a pathogen-associated molecular pattern (PAMP) that is recognized by toll-like receptor 5 (TLR5) expressed by monocytes and fibroblasts. The receptors at the surface of intestinal epithelial cells bind the conserved regions of flagellin (N and C terminals), resulting in the activation of cytokine secretion (YOON et al., 2012). Flagellin was found to be important for protective immunity by opsonic activity, resulting in the clearance of *C. chauvoei* by polymorphonuclear leukocytes in mice (TAMURA & TANAKA, 1984; TAMURA & TANAKA, 1987). Flagellar expression and motility are reversible in *C. chauvoei* and are associated with complete expression of virulence (TAMURA et al.,

1995). Further studies characterized flagellin and evaluated its protective activity by using a recombinant flagellin protein (KOJIMA et al., 1999; 2000). These authors reported poor protective immunity induced by the recombinant flagellin in mice, suggesting that a conformation-dependent epitope plays an important role in the development of immunity against blackleg. The poor protective activity of the recombinant flagellin protein observed previously (KOJIMA et al., 2000) can be attributed to the fact that these authors did not considered that there are a minimum of two copies of *fliC* gene on the chromosome of *C. chauvoei* (SASAKI et al., 2002). RYCHENER et al., (2017) found three copies of the allelic variants *fliC1*, *fliC2,* and *fliC3* of flagellin in most strains studied, thus showing 91.8% amino acid identity with each other in a given strain and 82-96% identity between the paralogues of different strains. THOMAS and collaborators (2017) also revealed the presence of three *fliC* genes.

The cell surface-associated antigens of *C. chauvoei*, other than flagellin, have not yet been explored. USHARANI et al., (2016) identified some important cell surface-associated proteins of *C. chauvoei*, such as enolase, chaperonin, ribosomal protein L10, flavoprotein, and glycosyl hydrolase, which showed protective antigenicity in other bacteria. However, further studies are necessary to evaluate the role of these surface-associated proteins in protection against blackleg.

Soluble antigens and toxins

The soluble antigens, mainly represented by toxins, are deeply involved in the pathogenesis of blackleg. At present, five *C. chauvoei* toxins are known: the hemolytic leukocidin CctA, oxygen-labile hemolysin D (or hemolysin III), DNase (β-toxin), hyaluronidase Nag (previously called γ-toxin), and neuraminidase/sialidase NanA.

The pore-forming, oxygen-stable leukocidin hemolysin called *C. chauvoei* cytotoxin A (CctA) confers strong hemolytic activity, which is observed as a halo around the colonies on blood agar growth medium (FREY et al., 2012). CctA as a mature protein has a molecular mass of 32.2kDa. It is a major toxin and hemolysin produced by *C. chauvoei*, which is shown to be highly cytotoxic to the bovine epithelial cell line ECaNEp (FREY et al., 2012). In addition, FREY et al., (2012) used the conventional assay for testing the potency of blackleg vaccine, which contains purified recombinant CctA as the sole antigen, and protects 80% guinea pigs from the challenge with virulent *C. chauvoei*. The antibodies directed against CctA play the main role

in the protective immunity exerted against blackleg; thus, it is a valuable candidate for blackleg vaccines and for the potency testing of current vaccines.

The previously described oxygen-stable necrotizing hemolysin (α-toxin) (HANG'OMBE et al., 2006) might be CctA, although the reported molecular mass of this α-toxin hemolysin is 25kDa, which is significantly lower. Alternatively, this α-toxin could represent the putative hemolysin III, also called hemolysin D or δ-toxin (protein #276) found on the genome of *C. chauvoei* (FREY & FALQUET, 2015), whose molecular mass is around 25kDa. However, the latter might correspond to a weak hemolysin that is oxygen labile and potentially thiol-activated (GILBERT, 2002). It must be noted that hemolysin III is not specific to *C. chauvoei* as several pathogenic, commensal, and environmental gram-positive bacteria express it or carry genes coding for this class of hemolysin. Although there is no clarity about hemolysin III in *C. chauvoei*, it is reported to be similar to the θ-toxin produced by *C. perfringens* and the tetanolysin produced by *C. tetani* (HATHEWAY, 1990). Using monospecific antibodies directed against CctA, FREY et al., (2012) fully neutralized all the hemolytic activity expressed by *C. chauvoei,* showing that other than CctA, this pathogen does not produce any entity with measurable hemolytic activity.

DNase (β-toxin) is an enzyme of the deoxyribonuclease type; it is a thermostable protein responsible for the nuclear degradation of muscle cells (HATHEWAY, 1990). This toxin actively participates in clostridial myonecrosis (CORTINAS et al., 1999). It was found in >80% *C. chauvoei* strains isolated from cattle (CARLONI et al., 2005), although the strains showed different capacities of toxin production. Full genome analysis of *C. chauvoei* revealed the presence of two genes encoding the large and small subunits of exo-deoxyribonuclease VII that most likely represent the DNase activity of *C. chauvoei* (FALQUET et al., 2013). The genes encoding exo-deoxyribonuclease VII are present and fully conserved in all 20 strains of *C. chauvoei* (RYCHENER et al., 2017).

Hyaluronidase (γ-toxin) is an enzyme inactivated by heat and capable of breaking down hyaluronic acid. It is assumed to be responsible for the destruction of the loose connective tissue that surrounds the muscles, thus favoring the spread of *C. chauvoei* in the tissues of the infected host (HATHEWAY, 1990). In addition, the end products of hyaluronate degradation are disaccharides, which might be a source of nutrients for the pathogen (HYNES & WALTON, 2000). The genome of *C. chauvoei* has two different hyaluronidase genes,

namely, *nagH* and *nagJ*. Currently, the functional activity of *nagH* has been confirmed (FREY & WÜTHRICH, unpublished data).

Neuraminidase/sialidase (NanA) was purified and characterized by HEUERMANN et al., (1991). They showed that the enzymatic activity cleaves N-acetylneuraminic acid (sialic acid) in carbohydrate polymers, present in many mammalian cell membranes as well as many microorganisms (USEH et al., 2003). NanA was characterized in detail as an 81-kDa protein that is secreted as a dimer (VILEI et al., 2011). It is encoded by the *nanA* gene, which is fully conserved across the *C. chauvoei* strains isolated over 60 years from various geographical locations across four different continents (RYCHENER et al., 2017). A recombinant molecule derived from *nanA* containing the sialic acid-binding domain (CBM40) is able to fully neutralize the sialidase activity of *C. chauvoei* (VILEI et al., 2011). Thus, NanA can also be used as a potential antigen to aid protective immunity.

The findings about the protective immunity exerted by soluble antigens against blackleg leave no doubt about the importance of these antigens in the pathogenesis of this condition. Interestingly, the sialidase *nanA*, hyaluronidase *nagH* and *nagI*, and leukocidin *cctA* genes are well conserved in *C. chauvoei* strains (VILEI et al., 2011; FREY et al., 2012; RYCHENER et al., 2017). Thus, the failures in vaccine development cannot be explained by the variations in the genes encoding major soluble antigens. A schematic illustration of blackleg pathogenesis, along with the major virulence factors, is shown in figure 1.

Pathogenesis

Although blackleg is a clinically well-known disease, there is currently no consensus on the mechanisms underlying the pathogenesis of *C. chauvoei*. *C. chauvoei* spores are found in cattle gut as well as in pasture soil, which indicates that the infection is acquired by the ingestion of *C. chauvoei* spores. The ingested spores or those produced after germination cycles in the gut are transported from the intestine or lesions in the oral cavity to the muscles and tissues by macrophages across Peyer's patches (JUBB et al., 1991; PIRES et al., 2012; UZAL, 2012). After reaching

Figure 1 - Schematic illustration of blackleg pathogenesis involving currently considered major virulence factors.
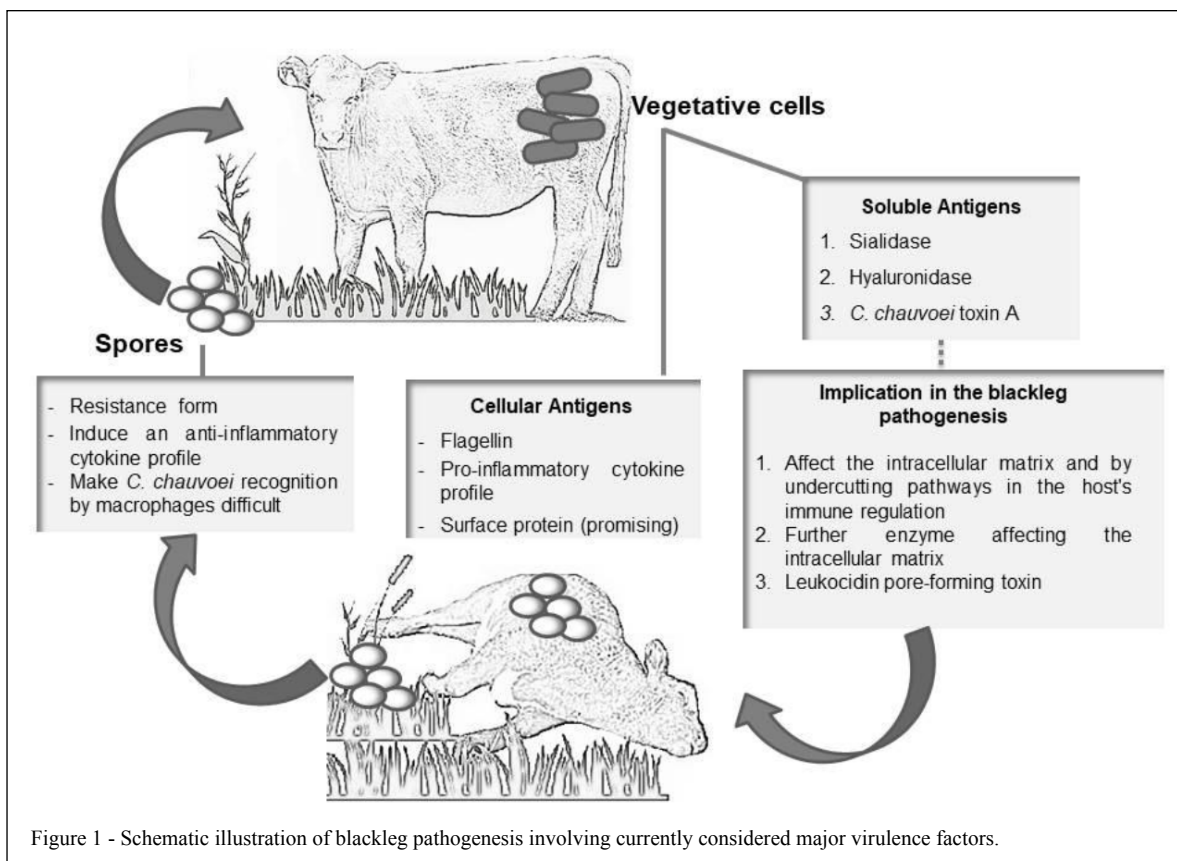
the tissues, the spores remain dormant until specific conditions are generated, such as anaerobiosis, resulting in their germination, multiplication, and consequently production of exotoxins (UZAL et al., 2012).

After a traumatic injury, the oxygen levels of the muscle tissue reduce, and the lactic acid concentration increases anaerobically during glycolysis (conversion of pyruvate to lactate), leading to the germination of spores, multiplication of bacteria, and consequent toxin production (MINETT, 1948a; UZAL et al., 2003). However, these hypotheses are not enough to explain why only young animals are affected or why the diaphragm or heart is the only affected area at times. In addition, it is not known whether the conditions allow the germination of latent spores in cases where there is no muscular injury, possibly because of the higher concentration of muscle glycogen caused by the high degree of muscle synthesis, which can serve as a substrate for *C. chauvoei* (VAN VLEET & VALENTINE, 2007). Latent spores of *C. chauvoei* can be found in healthy cattle carcass, in organs such as the liver and spleen (MINETT, 1948b; KERRY, 1964; SATHISH & SWAMINATHAN, 2009). In a surveillance study involving two slaughter houses of Sao Paulo, Brazil, *C. chauvoei* was identified by microbiological culture in 7.5% of muscle samples and 1.7% of liver samples (SCHOCKEN-ITURRINO et al., 2000).

A recent study showed that the vegetative and sporulated forms of *C. chauvoei* are able to resist the microbicidal effects of macrophages in murine and bovine monocyte-derived macrophages, supporting the importance of macrophages during the early pathogenesis of blackleg (PIRES et al., 2017). These authors also noted a pro-inflammatory cytokine profile such as IL-12 and IL-23 transcription in bovine macrophages after infection with vegetative *C. chauvoei*. Conversely, in bovine macrophages infected with the spores of *C. chauvoei*, an anti-inflammatory cytokine profile such as induction of IL-10 and TGF-beta transcription was observed (PIRES et al., 2017). The anti-inflammatory profile induced by spores might explain their latency after macrophage internalization. Future research should, thus, investigate the possible role of genetic susceptibility in the occurrence of blackleg. A starting point could be the genetic characterization of the ability of phagocytic cells, especially macrophages, to clear *C. chauvoei* post internalization.

Diagnosis

Very few affected animals survive, and death usually occurs within 48h of clinical manifestation. Once the animals die on the pasture, postmortem decomposition complicates an accurate diagnosis (DALY et al., 2009). A preliminary diagnosis of blackleg can be undertaken on the live animal on the basis of clinical signs and the presence of typical muscle emphysema. Postmortem findings include dark, discolored, swollen, and rancid muscle upon incision of the affected area. The affected muscle will contain excess fluid and gas bubbles and smell like rancid butter because of bacterial production of butyric acid. Body cavities will also contain excess fluid. Overall, tissue decomposition is rapid, assumingly by the action of potent toxins. In cardiac myositis, fluid accumulation is observed around the heart, with large amounts of fibrin too (SULTANA et al., 2008).

Traditionally, the diagnosis of blackleg is confirmed by microbiological culture and isolation of the causative microorganism. However, this is not always successful because of the difficulties in obtaining, submitting, and processing the samples in the laboratory. FARIAS et al., (2012) proposed the use of direct polymerase chain reaction (PCR) using common filter paper as an alternative to collecting, storing, and shipping material to the laboratory for the diagnosis of blackleg; the sensitivity and specificity of this approach was 100%. In addition, *C. chauvoei* is sensitive to oxygen, and it tends to be overgrown easily by other microorganisms in the samples (ASSIS et al., 2005).

Routine clinical microbiology laboratory tests might falsely identify *C. septicum*, instead of *C. chauvoei* (NAGANO et al., 2008). It is difficult to differentiate between *C. chauvoei* and *C. septicum* because of morphological and biochemical similarities. Based on 16S rDNA sequence analysis, *C. chauvoei* and *C. septicum* have been identified as closely related species with a high level of similarity (99.3%) (KUHNERT et al., 1996). THOMAS et al., (2017) also showed high relatedness (74%) between *C. chauvoei* and *C. septicum* by phylogenomic analysis. It should be noted that *C. septicum* is a microorganism capable of initiating malignant edema, a highly lethal exogenous infection (ASSIS et al., 2012). In humans, *C. septicum* is an important cause of death from spontaneous, nontraumatic gas gangrene in both adults and children (ABELLA et al., 2003; SMITH-SLATAS et al., 2006). BARNES et al., (1975) suggested that some of the deaths related to *C. chauvoei* are caused by *C. septicum* or a co-infection with *C. chauvoei* and *C. septicum*.

Further diagnostic methods for the identification and differentiation of *C. chauvoei* and *C. septicum* include immunofluorescence assays (SEISE et al., 2014), immune histochemistry (ASSIS et al., 2005), and MALDI-TOF

MS technology (GROSSE-HERRENTHEY et al., 2008). Molecular assays such as conventional PCR on the 16S rRNA genes (KUHNERT et al., 1997, 1996; UZAL et al., 2003), 16S and 23S rDNA spacer regions (SASAKI et al., 2001; IDREES et al., 2014) (SASAKI et al., 2001; IDREES et al., 2014), and flagellin genes (KOJIMA et al., 2001; SASAKI et al., 2002) and a multiplex real-time PCR (GAROFOLO et al., 2011; HALM et al., 2010; LANGE et al., 2010) have also been used as diagnostic methods. A recombinant flagellin-based ELISA assay was developed for the detection of *C. chauvoei* and could detect up to 10^4CFU/mL of *C. chauvoei*, in addition to its high specificity (USHARANI et al., 2015).

Prevention and control

Although published studies about the ability of vaccination to prevent morbidity and mortality by blackleg in cattle are scarce (UZAL, 2012), vaccination is the principal prophylactic measure that can be used to control the disease (CHANDLER & GULASEKHARAM, 1974). ARAUJO et al., (2010) evaluated the serological response of beef calf tissue subjected to different vaccination regimens. This study showed that booster shots significantly increased the serological response at 30 days post immunization. The serum IgG levels against *C. chauvoei* were significantly higher in calves that were first vaccinated at four months, compared to calves vaccinated at eight months of age. The serological response of calves that were vaccinated twice was found to be satisfactory, independent of the first vaccination being given at four or eight months of age. According to ARAUJO et al., (2010), cattle should be vaccinated at four months of age, followed by a booster dose one month later, and then annually repeated. For disease control, it is important to highlight that the carcasses of animals suffering from blackleg must be burned to restrain the contamination of pastures.

Some authors consider that the use of native strains can improve commercial vaccine preparations, by increasing bovine immune response (ORTIZ-ORTEGA et al., 2012). However, a recent study involving whole genome analysis of 20 strains of *C. chauvoei* isolated across four continents over 64 years (RYCHENER et al., 2017) indicated virtually no genomic variations between the strains used in vaccines and those obtained from outbreaks, suggesting that the reason for vaccine failure could be a factor other than the lack of similarity between the two sets of strains. Given that vaccination is quite common, and so are disease outbreaks, essential questions continue to remain about the causes for vaccine failure. Most likely, practical issues such as management failures, inconsistent vaccination, delayed application of the first dose, incorrect timing of booster doses, and vaccination of dams are the causes for vaccine failure. In addition, cattle breeders tend to stop vaccinating herds when the outbreaks reduce, and as a result, the disease recurs.

Because *C. chauvoei* survives within macrophages, inactivated vaccines might not elicit the best type of immune response (cellular immunity). In Brazil, a live attenuated vaccine was patented by the Institute Oswado Cruz in 1908 (GODOY, 1910). This was the only live vaccine against *C. chauvoei* available in the world. However, since 2014, the production of this vaccine was stopped despite personal observations that this widely used live vaccine promoted adequate protection against blackleg in Brazil.

The potential underestimation of blackleg incidence is a limiting factor for prevention and control measures. Farmers form an important bridge between the cases of blackleg and official surveillance programs. Offering compensation for reporting a case might motivate the farmers to report every case. Additionally, a subsidized vaccination program could help control blackleg occurrence and thus reduce environmental contamination with *C. chauvoei* spores, especially in high-risk areas. Alternative measures to prevent and control blackleg can focus on specific pasture management practices, like artificial drainage of pastures. In fact, a recent study pointed out that blackleg cases are usually clustered within geographic areas with poor water permeability (WOLF et al., 2017).

CONCLUSION

Blackleg is an acute and often fatal infection occurring in cattle that continues to remain endemic worldwide despite large vaccination programs. Studies characterizing cellular and soluble antigens are necessary to improve the chances of developing a protective vaccine. We also highlighted that the commercial vaccines are bacterins that are probably ineffective in extending immunity against *C. chauvoei* spores, sialidase, and CctA.

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DECLARATION OF CONFLICT OF INTEREST

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