Review

Blackleg: A Review of the Agent and Management of the Disease in Brazil

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Blackleg: A Review of the Agent and Management of the Disease in Brazil

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Abstract: The genus Clostridium is an important group of pathogenic and nonpathogenic Gram-positive anaerobic bacteria with sporulation capacity and wide distribution in different environments, including the gastrointestinal tract of healthy and diseased animals and humans. Among the pathogenic species of the genus, *Clostridium chauvoei* stands out as a histotoxic agent. It causes important myonecroses such as blackleg, a disease with high lethality and mortality, especially in young cattle, and is responsible for significant losses to livestock worldwide. The pathogenicity of the disease is complex and has not yet been fully elucidated. The hypotheses cover processes from the initial absorption to the transport and deposition of the agent in the affected tissues. The virulence factors of C. *chauvoei* were divided into somatic and flagellar antigens and soluble antigens/toxins, which are the main antigens involved in vaccines against blackleg in Brazil and worldwide. This review provides important information on the first and current approaches to the agent *C. chauvoei* and its virulence factors, as well as provides a compilation of data on Brazilian studies related to blackleg.

Keywords: *Clostridium chauvoei*; toxin-host interaction; virulence factors; myonecrosis; vaccination

1. Introduction

The genus Clostridium comprises more than 200 species of bacteria, most of which are nonpathogenic and live in the environment, plants, skin, and mucosa of healthy and/or diseased animals and humans (especially in the gastrointestinal tract). Some of these species contaminate and grow in foods of plant and animal origin, causing deterioration [1]. These are strictly anaerobic bacteria, although the degree of tolerance to the presence of oxygen is particular to each species. Clostridia are usually Gram-positive rods that, under adverse conditions, such as the absence of nutrients and presence of oxygen, can take on a resistant morphology called spores [2].

Between 40 and 50 species are associated with clinical conditions in domestic animals and humans. Most pathogenic species, about 30, are considered secondary pathogens. Only about 15 of them are thought to be important primary pathogens [1,3]. The main pathogenic species cause disease through the production of exotoxins, which are responsible for the development of lesions and clinical signs. These toxins are among the most potent of microbial origin and are still considered the main basis for the identification and differentiation of the various pathogenic species of Clostridium. Despite the small number of pathogenic microorganisms, clostridia are responsible for the production of 20% of the main and most potent toxins of bacteriological origin [3].

Clostridial toxins are a wide variety of proteins with different sizes, structures, and mechanisms of action. They also differ in their ability to diffuse in the host organism and in the site of action. Some act locally, while others enter the bloodstream, spreading to different organs and tissues. However,

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in some clostridioses, the toxins and the multiplication of the agent are equally important in the pathology, such as in myonecroses [4,5].

Many clostridia cause diseases of public-health importance through the action of their toxins. These proteins trigger clonic signals, which are related to certain types of clostridiosis. Examples that should be considered are the pseudomembranous colitis of *C. difficile*, the clonic paralysis of the infection by *C. tetani*, and the flaccid paralysis of *C. botulinum* [6]. Other clostridia, although producing totally different toxins, cause diseases with similar clinical signs. Myonecroses, for example, can be caused by the following species alone or together: *C. septicum*, *C. chauvoei*, *C. novyi* type A, *C. perfringens* type A, and *C. sordellii*, but the lesions are similar regardless of the etiology of the disease. Despite the clinical and pathological similarities, each Clostridium species has its own mechanism that culminates in the observed changes. Thus, few generalizations about the mechanisms of virulence can be made, requiring the study and individualized understanding of the various species [7].

The present article aims to review the main virulence factors and parasite/host ratio of *C. chauvoei* and provide a compilation of data on Brazilian studies of blackleg.

2. Myonecroses

Histotoxic clostridia are pathogens common to humans and animals that can cause two forms of necrotizing myositis, usually fatal, called gas gangrene and blackleg. Gas gangrene or malignant edema is an exogenous infection that affects practically all species of veterinary interest, such as cattle [8,9], sheep, goats [10], pigs [11], horses [12], dogs [13], and birds [14]. Caused by one or more of *C. septicum*, *C. chauvoei*, *C. novyi* type A, *C. perfringens* type A, and *C. sordellii*, gas gangrene requires close contact between these agents and domestic animals. This is favored by the contamination of wounds resulting from surgical practices and even vaccinations without aseptic care [2,15].

Unlike gas gangrene, blackleg is an endogenous infection that affects only domestic ruminants, especially cattle aged 4-6 weeks, and results from the activation of latent *C. chauvoei* spores present in the muscle [7,16,17]. This is considered an exclusively extracellular bacterium [16]. The pathogenesis of this disease is not well understood. The main hypothesis is that this agent is absorbed from the intestinal mucosa and deposited in the muscle after transport via the blood circulation by tissue macrophages [16,18]. To test this hypothesis, Pires et al. [15] evaluated the survival of vegetative cells and spores of *C. chauvoei* after phagocytosis by a macrophage cell line of murine and bovine macrophages and demonstrated that both remain viable after internalization by these phagocytic cells. These data support the hypothesis that macrophages play an important role in the pathogenesis of blackleg.

The factors responsible for the germination of spores in the muscles have not yet been fully established; it is believed that traumas, especially in large muscle masses, create an environment conducive to germination and consequent production of toxins, which usually culminate in rapid muscle necrosis and death of affected animals [18,19].

3. *Clostridium chauvoei*

Among the pathogenic microorganisms of this genus Clostridium, *C. chauvoei* stands out as a particularly important microorganism in veterinary medicine. The etiological agent of both blackleg and gas gangrene, this bacterium has been responsible for considerable economic losses to livestock worldwide [16,20]. Although gas gangrene and blackleg have been described since the mid-19th century, *C. chauvoei* was only described in 1887 [21]. Certainly, the identification of the agent was delayed due to the great complexity of the bacteriological isolation, an obstacle that holds true today. Several factors are involved in this diagnostic difficulty, including the specific growth requirements of the bacterium and the fastidious growth of this pathogen [22,23]. In addition, clinical samples are often contaminated with other anaerobic bacteria that spread after the death of the animal [24]. The contaminating microorganisms are less demanding and grow faster than *C. chauvoei*, inhibiting its growth [25].

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Like most clostridia, *C. chauvoei* is a Gram-positive, motile rod measuring 0.6 × 3-8 μm, occurring singly or in pairs [20]. The bacterial spores are oval, central to subterminal, causing deformation of the mother cell. Cultivation on solid media allows the production of colonies with different morphotypes, including regular shapes of mother-of-pearl buds or irregular colonies with denture edges and a rough surface. Identification can be facilitated by culturing on sheep blood agar, as it results in colonies surrounded by extensive hemolysis [18,22].

Thus, the differentiation of *C. chauvoei* from other clostridial species by means of culture is extremely difficult. In addition to the various forms of growth, carbohydrate fermentation is nonspecific, and the use of complementary laboratory tests is essential [25], such as direct immunofluorescence [26,27] and PCR [13,28].

C. chauvoei is a widespread microorganism, especially in cattle production areas such as pastures and confinement pens [29,30]. This agent contaminates the soil, pastures, drinking troughs and pens through the decomposition of carcasses and the excretion of spores in the feces. Once contaminated, the area can remain contaminated for years [25,31].

The maintenance of viable spores in the environment, which can return to the vegetative state if they detect ideal conditions for germination, is the most significant factor for the infection of animals. Ingestion of spore-contaminated pasture and water is the main route of transmission, as direct animal-to-animal transmission does not occur. Environmental contamination allows the perpetuation of the agent, especially in farms where the disease is endemic [17,32,33]. Animals infected with *C. chauvoei* may exhibit loss of appetite, high fever, myonecrosis, swollen lesions, and lameness [34,35]. The rapid course of the disease makes the early identification and treatment of these clinical signs difficult, indicating vaccination is the best alternative for prevention of blackleg.

Despite its recognized importance as a pathogenic agent for domestic animals, especially cattle, few studies have been conducted to better characterize the agent and its mechanisms of virulence.

4. Virulence factors

Moussa et al. [36] divided the *C. chauvoei* antigens into two groups: cellular antigens and soluble antigens. Cellular antigens are classified into somatic and flagellar antigens, while soluble antigens encompass mainly toxins. Soluble antigens are directly involved in the pathogenesis of blackleg [17].

4.1. Soluble antigens

Until recently, it was assumed that this clostridium produces at least five toxins: an oxygen-stable hemolysin (alpha), a DNase (beta), a hyaluronidase (gamma), an oxygen-sensitive hemolysin (delta) [19], and a neuroaminidase [37]. However, Frey et al. [38] described a new toxin whose presence is currently considered essential for the occurrence of the disease as well as for adequate immunization of animals [17,39]. This toxin was named CctA.

4.1.1. Alpha toxin

Alpha toxin was first studied by Moussa et al. [36] and Verpoort et al. [40], who reported some activities and biological characteristics from nonpurified *C. chauvoei* culture filtrates. This exotoxin is a 27-kDa hemolysin that was later characterized as a necrotizing, hemolytic, and lethal protein [41].

The alpha toxin was purified and partially characterized for the first time by Tamura et al. [42]. This research group demonstrated that the production of toxin peaks in the logarithmic phase of bacterial growth and that the erythrocytes of sheep, cattle, and birds (chickens) were susceptible to the hemolytic action of this protein, while the erythrocytes of goats, rabbits, and guinea pigs were classified as partially resistant and those of horses as resistant. Hang'ombe et al. [41] also analyzed the sensitivity of erythrocytes from different animal species and found results much like those of Tamura et al. [42], the only difference being in the sensitivity of sheep and bovine erythrocytes. It is speculated that the variation in sensitivity between species may be explained by the existence or absence of membrane receptors necessary for the binding of the toxin [41]. These same authors also found that the higher the temperature, the lower the amount of toxin required to achieve hemolysis

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of 50% of the erythrocytes, indicating that the alpha toxin has its action dependent on temperature. However, to date, no study has been able to fully elucidate the mechanism of action of *C. chauvoei* alpha toxin [41]. As there have been few conclusive studies on the toxins of *C. chauvoei*, alpha toxin was long considered the main toxic factor of this species of Clostridium [19].

4.1.2. Beta toxin

Beta toxin, or DNase, is a deoxyribonuclease-type enzyme [43] found in more than 80% of *C. chauvoei* strains [44]. It is a 45-kDa thermostable protein responsible for nuclear degradation in muscle cells [19] and actively participates in cases of clostridial myonecrosis [45]. Although there is no correlation between the production of alpha and beta toxins [31], both are considered fundamental in the process of gangrenous myositis triggered by this microorganism [44]. A study analyzed the complete sequences of 20 *C. chauvoei* strains isolated from different continents for 64 years and found the presence and conservation of two genes that are likely involved in DNase activity [17,46].

4.1.3. Toxin range

Hyaluronidase is a general term for enzymes that can digest mainly hyaluranate, present in hyaluronic acid and hyaluronan. Hyaluranate is a linear polymer of nonsulfated glycosaminoglycan found in many tissues and body fluids of higher organisms and is a major constituent of soft connective tissue, muscle, and skin. Therefore, pathogens that produce hyaluronate, which are most if not all Gram-positive bacteria, can cause infections initiated in the mucosa, subcutaneous tissue, and/or skin [47]. It is noteworthy that the genome of *C. chauvoei* has two different hyaluronidase genes: *nagH* and *nagJ* [17].

In the pathology of blackleg and gas gangrene, although gamma toxin, a hyaluronidase, is not considered a lethal toxin, it is believed to be responsible for the great disorganization of muscle tissue, with loss of almost all structures [43]. Normally, during an infection of any type, the connective tissues and the skin provide a defense mechanism against infectious agents, resisting the penetration of these pathogens. However, hyaluronidase-producing bacteria, such as *C. chauvoei*, can weaken the restrictions imposed by the constitution of connective tissues, destroying them and facilitating their propagation. In addition, the degradation products of hyaluronate are disaccharides that can be a source of nutrients for a pathogen [47].

4.1.4. Delta toxin

Delta toxin is a thiol-activated cytolysin [48]. It is sensitive to the presence of oxygen, whose presence considerably reduces its hemolytic action. There is no in-depth characterization of delta toxin, and it is only known that it is similar to perfringolysin O produced by *C. perfringens* and tetanospasmin produced by *C. tetani* [19]. Thiol-activated cytolysins are so called because they act mainly on cell membranes with high cholesterol content. After anchoring in cell membranes, these toxins oligomerize, forming pores with up to 50 toxin subunits [49].

4.1.5. Neuraminidase

Also called sialidases (*NanA*), neuraminidase belongs to a class of glycosyl hydrolases that release the terminal *N*-acetylneuraminic or sialic acid residues of glycoproteins, glycolipids, and polysaccharides. Neuroaminidase-type toxins have been detected in a variety of microorganisms, such as viruses, bacteria, and protozoa [37]. Likewise, *C. chauvoei* is known to produce neuraminidases that play a significant role in the pathogenesis of the infection [50].

Neuraminidases are responsible for the cleavage of sialic acids in infected tissues and the hydrolysis of erythrocytes, facilitating the propagation of the pathogen and the disease. Active sialidases are composed of three domains: the N-terminal portion, which appears to be responsible for binding to its receptor; the central portion, which binds sialic acid; and a C-terminal enzymatic portion. It is a toxin of chromosomal origin encoded by the *nanA* gene, which is found in a wide variety of strains of *C. chauvoei*, including the reference sample ATCC 10092. The *nanA* gene

determines the expression of the protein as a 150-kDa dimer, which by proteolytic cleavage results in a metabolically active protein of 65 kDa, which presents a similarity of 82% with the sialidase produced by *C. septicum* [51].

The sialidase isolated from *C. chauvoei* is a highly stable dimeric protein of 135 kDa. When it was put through five cycles of freezing and thawing, it showed no loss of activity. The activity was maintained at 37°C in buffer C for at least 1 h [50]. This toxin remains active over a wide range of temperatures (4-50°C) and pH values (4-7.5), with an optimum at 37°C [33]. The main substrates of *C. chauvoei* sialidase are glycoproteins, which exist in large amounts in muscle tissue and in the cell wall of erythrocytes, which contributes to the hydrolysis of these cells and tissues. This suggests that even under apparently unfavorable conditions, the toxin remains active, being able to support the growth and spread of the bacteria to cause muscle damage [50].

4.1.6. Toxin A (CctA) of C. *chauvoei*

Frey et al. [38] described and initially characterized a new toxin produced by *C. chauvoei*: Toxin A (CctA) is a 33.2-kDa protein belonging to the beta-barrel family of pore-forming toxins within the leukocidin superfamily. The sequence analysis of the CctA gene showed great similarity with the alpha-hemolysin genes of *C. botulinum* (50% identity and 80% amino acid similarity) and with the NetB of *C. perfringens* (44% identity and 60% amino acid similarity) and *C. perfringens* beta toxin (33% identity and 51% amino acid similarity).

Roughly one-third of the many clostridial toxins, as well as many other bacterial toxins, are classified as pore-forming [3]. Beta-barrel pore-forming proteins are characterized by having a conserved structure that includes domains that bind to specific receptors on target cells, followed by internalization of part of the hydrophobic amino acid sequence in the host cell membrane that allows the anchoring of the protein. After binding and anchoring of the protein, oligomerization occurs, usually into heptamers that form pores, which culminates in a large influx of extracellular content and consequent lysis of the affected cells [52].

The gene encoding CctA is conserved, having been identified in strains of isolates on different continents, as well as in the reference sample of the ATCC (10092). Although the toxins described above were believed to be responsible for the symptoms of anthrax and gas gangrene, specific antibodies against CctA protected 90% of the challenged animals, neutralizing all the cytotoxic and hemolytic effects promoted by the supernatant of *C. chauvoei* cultures [38]. These results are considered quite promising because this is the first time that vaccination with an exotoxin produced by *C. chauvoei* has protected such a high number of challenged animals.

4.2. Cellular antigens

According to Robert [53], cellular antigens of *C. chauvoei* can be subdivided into somatic antigens and flagellar antigens. The somatic antigens are related to bacterial cells and include agglutinogenic antigen, heat-stable O antigen, nonagglutinogenic antigen, and heat-labile antigen that does not present the O and HO antigens. In turn, flagellar (H) antigens are thermolabile, and two distinct ones have been identified. Analyses of these antigens in different strains of *C. chauvoei* revealed that the somatic antigen is common to all of them, while one of the flagellar antigens differs between samples isolated from cattle and sheep [54]. Somatic antigens are considered essential immunogens linked to protection against *C. chauvoei* and are present in past and present vaccine formulations [17,55].

4.2.1. Flagellar antigens

Bacteria move along chemical gradients using one of the smallest and most complex motors in the biosphere, the flagellum. This structure is practically identical in Gram-negative and Gram-positive bacteria, except that the former emerges from a second outer membrane, which is absent in Gram-positive bacteria. Flagella also aid in sensory function, allowing the bacterium to respond to chemical stimuli and avoid unfavorable environments, such as extremes of pH and high salt concentrations [56].

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Flagella consist of a long helical filament composed of flagellin polymers, which emerges from a "hook" connected to a basal body that is anchored inside the cell membrane. There is only one study on a basal model of the structure of the flagellum of *C. chauvoei*, which according to Hamilton & Chandler [57] would look like that of other Gram-positive bacteria. Regarding the amino acid composition, the flagellin of *C. chauvoei*, which is encoded by the *fliC gene,* is similar to the flagellins of *Salmonella* Typhimurium, and cysteine and tryptophan are not detected in its constitution. It has low percentages of proline, methionine, tyrosine, and histidine [58].

In general, bacterial flagella are best known for their role in bacterial motility. However, for certain pathogenic bacteria, these structures also seem to be an important aspect in the interaction of the parasite with the host. Flagellated strains of *C. chauvoei* are more virulent than naturally nonflagellated strains [59]. Despite the constitutional similarity, the flagella of *S.* Typhimurium are more involved with the invasive capacity of the strain than with its virulence, a fact that limits further comparisons.

While the structure and interaction between the *C. chauvoei* flagellum and host cells have not been well characterized, the immunogenic potential of this structure has been widely analyzed. In contrast to the other clostridia in which immunity against toxins has always been considered the predominant form of protection, immunity against *C. chauvoei* was considered for many years to be exclusively antibacterial [60]. The immunogenicity of the flagellum of *C. chauvoei* has been the subject of several studies. Techniques ranging from protein purification [61] to gene recombination [62] have been tested, but unfortunately, the results are controversial. Over the years, the only consensus regarding this protein has been that it is an important mechanism of virulence and immunogenicity, but how and how much cannot yet be specified.

5. Prevention, control, and eradication

Vaccination against several clostridial pathogens, including *C. chauvoei*, has been used as a prophylactic measure worldwide for more than 70 years [63]. In particular, vaccination against *C. chauvoei* is one of the main sanitary management measures. Scientific evidence of the effectiveness of vaccination, as well as of the antigens adopted, in preventing the disease and deaths caused by it is still scarce [16]. Several antigenic compositions have been proposed. The first studies on immunogens sought to establish whether the best vaccine formulation should be based on bacterins, toxoids, or both. Perhaps, due to the difficulties in cultivation, it was initially proposed that immunity was conferred only by the bacterins that then composed the commercial antigens [55,64]. Efficient toxoids were only obtained in laboratory formulations, with controlled production conditions associated with the concentration of the toxins before the inactivation step [65]. Vaccine formulations were also evaluated in sheep by Coackley and Weston [66]. These researchers immunized three groups of sheep with toxoid, bacterin, or bacterin + toxoid. Only sheep vaccinated with toxoid died after challenge, which reinforced the initial idea that the immunogens should be based on bacterins. These authors proposed that vaccinated animals would be protected for at least 18 months after vaccination.

As the bacterium had been considered one of the most important antigenic components, we sought to determine which would be the most antigenic portion of the bacterial cell, the cell wall or the flagellum [67]. We concluded that both could bring about a protective response. These antibodies are important in the opsonization of pathogens, increasing the efficiency of phagocytosis and protecting rats from experimental infection with *C. chauvoei* [68].

With the discovery of CctA [38], new perspectives in the study of prophylactic methods for blackleg have emerged. Researchers have performed the potency test recommended by the European Pharmacopeia using a CctA recombinant toxin and observed that all vaccinated animals survived the challenge. Thus, the new toxin is expected to confer protective immunity to animals, and the recombinant protein technique would facilitate the production of the immunogen.

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6. Blackleg in Brazil

The occurrence of blackleg in Brazil dates to the 20th century. In 1905, the Ministry of Justice instructed the Oswaldo Cruz Institute to discover an effective way to minimize the losses caused by the disease, which had decimated the herds of São Paulo and Minas Gerais. It was in 1906 that Alcides Godoy, a scientist at the Institute, discovered the first veterinary vaccine in the country for the prophylaxis of infectious-contagious diseases: the vaccine against blackleg, better known at that time as Peste da Manqueira [69]. The immunogen produced was patented, and its patent was renewed after 15 years for another 15 years. The rights derived from the sale of the "Manguinhos" anti-southern disease vaccine were assigned to the Institute on the condition that the respective income would be applied to the scientific activity of the Institute. The disease was such a bottleneck for Brazilian livestock at that time that the sale of this vaccine was one of the institution's main sources of income, enabling studies on Chagas disease, yellow fever, and leishmaniasis [70].

With the advent of vaccination, the disease came under control, so it was no longer the object of interest of Brazilian researchers. However, there remains great interest among ranchers in protecting their herds by vaccinating them regularly and public agencies in ensuring the quality of these immunogens. This contributes to the fact that clostridial vaccines are among the most sold vaccines in Brazil [2]. Despite the significant reduction in the incidence of this disease, outbreaks and isolated cases associated with nonvaccination of animals are still described [28,71], and there are few reports of successful treatment [8].

In Brazil, there are at least three strands of study regarding blackleg: those related to the production of vaccines and vaccination protocol of animals, methods for evaluation of antibody production, and diagnostic methods of the disease.

7. Production of vaccines and vaccination protocols of animals against blackleg

Despite the massive immunization of the Brazilian herd, the occurrence of blackleg is still an obstacle for Brazilian livestock. According to Assis-Brasil et al. [72]. Blackleg is the second most common disease detected in calves aged between four and six months. This led some researchers to question the efficiency of the strain used as a reference in immunization. Previous studies indicated a difference in the protective immunity conferred by the vaccination of animals with different strains against challenge with heterologous samples [8,60].

Given this information and the recurrent occurrence of anthrax in vaccinated herds, some studies have compared the MT vaccine strain with local samples. Guinea pigs vaccinated with commercial products and challenged with the MT sample did not exhibit protective immunity with 95.46% of the tested vaccines. On the other hand, when a local strain was used instead of the vaccine sample, only 36.36% of the vaccines were approved [73]. Similar results were also reported by Araujo et al. [74]. There is no consensus in the national or international literature on this subject. Other researchers found no difference in the immunity conferred by different strains of *C. chauvoei*. According to these authors, any vaccine failures have causes unrelated to the efficiency of the vaccines [75].

Despite the lack of consensus on the ideal composition of immunogens against blackleg, vaccination against *C. chauvoei* is part of the health management of Brazilian livestock. It is recommended that cattle be immunized between three and six months of age, with a booster every 30 days. After that, vaccination should be annual [76]. In places of high prevalence, the vaccination interval should be reduced to nine months [77].

On this topic, some studies were conducted in Brazil to establish the importance of passive immunity and the best protocol for vaccination of calves. According to Araújo et al. [78], passive immunity can be detected in calves up to three months after receiving colostrum from cows vaccinated up to 30 days before calving. The authors also emphasize that there is a positive correlation between the antibody titer observed in the calves and the time of vaccination of the cow. Thus, the closer to calving the cows received the immunization, the higher the antibody titer of the calves at three months of age.

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Regarding active immunity, Araújo et al. [74] evaluated the serological response of calves subjected to three different vaccination protocols against anthrax: animals vaccinated at four and eight months of age; vaccinated at eight months with a booster 30 days later; and those who received a single dose of the vaccine at eight months of age. Using enzyme-linked immunosorbent assay, the authors concluded that only the last vaccination schedule would not guarantee satisfactory antibody titers for the vaccinated animals when they were 10 months old.

8. Diagnosis

In Brazil, despite the significant number of foci, most diagnoses are based only on inconclusive clinical signs and/or lesions at necropsy, with few reports of laboratory confirmation. It is noteworthy that the etiological diagnosis of blackleg and gas gangrene caused by *C. chauvoei* should be carefully addressed by veterinarians and laboratory technicians, given that this microorganism is a frequent invader of carcasses [2].

Bacterial isolation usually requires time-consuming and expensive laboratory procedures and trained personnel [22]. Direct immunofluorescence, the gold-standard diagnostic method, has been used worldwide and is considered a fast and safe technique. It does require a special microscope, primary antibodies labeled with fluorochromes, and fresh or specially processed material [27].

An alternative for the diagnosis of this disease is immunohistochemistry, which, by combining histological, immunological, and biochemical techniques, allows the localization of tissue components in situ by labeling with specific antibodies and marker molecules. The advantage of this technique is the use of formalin-fixed materials, allowing for long times between collection and laboratory processing without interfering with the diagnosis [79]. These long possible storage periods for any clinical material are highly relevant in veterinary medicine because most rural properties are far from diagnostic and research centers. In addition, the fixation of the tissues prevents their autolysis, preventing saprophytic clostridia from multiplying. All these factors increase the accuracy of the etiological diagnosis, information that allows the adoption of appropriate immunization schedules, in addition to serving as a reference for the vaccine production industry [7,80,81].

Polymerase chain reaction stands out as a viable technique for the identification of microorganisms because it is extremely versatile, being able to identify the agents in cultures, fresh clinical specimens, or formalin-fixed and paraffin-embedded tissues [13,82]. It has the disadvantage of identifying the agent without colocation with the wound.

9. Conclusions

Clostridium chauvoei is the etiological agent of blackleg, a myonecrosis highly important in veterinary medicine due to the heavy losses of livestock worldwide due to its high lethality and mortality in ruminants, especially cattle. The rapid course of the disease and its pathogenesis are not yet fully understood, making vaccination the main approach for control and prophylaxis used in Brazil and worldwide. Although the main virulence factors of *C. chauvoei* have been evaluated and described, studies are still needed to better characterize the parasite/host relationships of these antigens so that they can be applied to the development of new vaccine formulations, which we hope will improve the protection of *C. chauvoei*–immunized animals through simplified production strategies and lower costs when compared to conventional vaccines.

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